Effectiveness of Bulb Extracts of the Genus *Allium*

Dissertation

zur

Erlangung des Doktorgrades

der Naturwissenschaften

(Dr. rer. nat.)

dem

Fachbereich Pharmazie der

Philipps-Universität Marburg

vorgelegt von

M.Sc. Sahar Samadi

aus

Tehran, Iran

Marburg/Lahn 2018

Erstgutachter: Prof. Dr. Michael Keusgen

Zweitgutachter: Prof. Dr. Klaus Reuter

Eingereicht am 24 May 2018

Tag der mündlichen Prüfung am 11 July 2018

Hochschulkennziffer 1180

Preface

This thesis is the result of a research project that I carried out at the institute of pharmacy at Philipps-Universität Marburg. During this research I received academic and practical supports from different persons whom I would like to acknowledge here.

First of all, I would like to express my sincere gratitude to my advisor Prof. Dr. Michael Keusgen for his continuous support, patience, motivation, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis.

Next, I would like to thank my thesis committee: Prof. Dr. Maike Petersen, Prof. Dr. Moritz Bünemannand and Prof. Dr. Klaus Reuter for taking the time to review this work and also for their insightful comments.

I would like also to thank my colleagues and fellow lab mates for the stimulating discussions, for working together before deadlines, and for all the funs we had together.

I wish also to acknowledge the role of my parents and my sister for their spiritual supports and their blessings throughout this research.

Finally, I would like to acknowledge the most important person in my life – my husband Meysam, who has been a constant source of strength and inspiration. I can honestly say that it was only his determination and constant encouragement that ultimately made it possible for me to see this project through to the end.

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List of abbreviation

amu	Atomic mass unit
Acc. No.	Accessions-number
CSO	Cysteine sulfoxides
°C	Degree celsius
DMSO	Dimethyl sulfoxide
Extr.	Extract
EtOAc	Ethyl acetate
G	Gram
hr	Hour(s)
HPLC	High performance liquid chromatography
HR	High resolution
IPK	Institut für Pflanzengenetik und Kulturpflanzenforschung
L	Liter(s)
M	Meter
Min	Minute
mL	Milliliter(s)
MS	Mass spectrometer/Mass spectrometry
µ	Micro
NMR	Nuclear magnetic resonance
PDA	Potato dextrose agar
RT	Retention time
S	Second
subg.	Subgenus
ssp.	Subspecies
SD	Standard deviation
TAX. No.	Taxonomic number
UV	Ultra violet
VCOs	Volatile compound
ZOI	Zone of inhibition

Abstract

Allium species like garlic (*Allium sativum*) and kitchen onion (*Allium cepa*) have been used since ancient times as vegetable, spice and remedy for different diseases. For these purposes, usually their bulbs and green plant are of interest. Wild *Allium* species have also been used in a similar manner. The antimicrobial activity of these plants is believed to be related to their allicin content, other thiosulfinates, and their sulphuric transformation products. In this thesis, we investigate antifungal activities of 51 *Allium* spp. belonging to 9 subgenera against 4 plant pathogenic fungi and 3 human pathogens (dermatophytes). The plant pathogenic fungi under considerations are *Mucor hiemalis, Aspergillus flavus*, *A. niger* and *Penicillium italicum*. The investigated human pathogens are *Epidermophyton floccosum*, *Cryptococcus neoformans* and *Basidiobolus ranarum*. To this end, we test dilution series of ethyl acetate extracts obtained from *Allium* bulbs on the above-mentioned fungi using three different methods as (i) potato dextrose agar (PDA) micro-dilution susceptibility testing, (ii) disk diffusion method, and (iii) double-dish chamber method. We show that among the tested *Allium* samples, *A. sativum* from the subgenus *Allium*, with the total average MIC of 0.25mg/mL, represents the highest anti-fungal activity against all the tested human and plant pathogenic fungi. In addition, our investigation reveals that *A. stipitatum* belonging to the subgenus *Melanocrommyum* with a total average minimum inhibitory concentration of 0.55mg/mL exhibits a significant effect towards all the pathogens. We also show that *E. floccosum* has the highest susceptibility, while *P. italicum* demonstrates greater resistance towards *Allium* extracts and miconazole. Our results indicate that extractions of *Allium* spp. have antifungal activity and might be promising not only in 'biological' treatment of fungal-associated plant diseases, but also as natural remedies in order to cure fungal skin infections. The effect seems to be partially related to volatile sulphur compounds, and other compounds like saponins and flavonoids must be considered.

1. INTRODUCTION

1.1 History of natural products as medicines

Natural products are small molecules produced by a living organism that has an interesting biological or pharmacological activity as well as chemical structure [1]. The very first reported natural product usage goes back to Egypt, 2900 B.C. Over 700 plant-based drugs ranging from pills, gargles to ointments and infusions were documented on the Ebers Papyrus. Mesopotamia (2600 BC) is also considered as an ancient place for using natural products, proving the use of *Cupressus sempervirens* (Cypress) and *Commiphora species* (myrrh) to treat cold, cough and inflammation [2]. The very first records of the natural product usage in China is documented in 3 records from 1100 B.C. to 650 A.D., containing the usage information of around 1200 drugs and prescriptions. Dioscorides, a Greek physician, also recorded the collection, storage and the uses of medicinal herbs back in 100 A.D. [2]. The Arabs had a great influence on the history of the natural product by preserving the Chinese and Indian as well as Greco-Roman knowledge of herbal medicine and expanding it throughout the world. The first privately owned pharmacies belonged to Arabs in the 8th century. And Avicenna, a Persian pharmacist, who contributed much to the sciences of pharmacy and medicine, wrote the Canon of Medicine, an encyclopedia of medicine that remained a medical authority for centuries [2].

1.1.1 Natural products from plant sources

As explained in the history of natural products, a human being has always been in search for healing powers in plants and has experimented for centuries by trial and error to find out the uses of medicinal plants, from discovering a cure to a deadly disease to search for foods or spices [3-5]. Evidence has shown that this goes back to around 60.000 years ago when Neanderthals living in present-day Iraq knew about the benefits of hollyhock (*Alcea* sp.) [6, 7]. The medicinal uses of plants have been very well documented for thousands of years [8, 9]. However, the isolation of the active compound was first done by Friedrich Sertürner from *Papaver somniferum*, by the isolation of morphine at the beginning of the nineteenth century, and since then natural products have been broadly screened for their medicinal purposes [10].

By producing structurally diverse secondary metabolites, plants have adapted themselves over millions of years to tolerate insects, fungi, and bacteria [8, 9]. Although these compounds are mainly being used as weapons by plants, they have been proved to have other functions like metal transporting agents, sexual hormones, differentiation effectors and etc. [11]. Plant secondary metabolites, best known as natural products, are believed to be the best source of potential drug discovery [2, 12-15]. Based on a report published by WHO (World Health Organization), for primary health care still, 80% of the people rely on plant-based traditional medicine [16]. Until now only 10% of the whole biodiversity has been evaluated for potential biological activity, which means still many useful compounds is waiting to be discovered [2]. It should be acknowledged that the structural diversity of the natural products is quite unique in comparison to the standard combinatorial chemistry [2]. So traditional medicine not only has led to the isolation of many natural products, but it has also promoted further investigations of medicinal plants as potential medicines [17]. Many of the natural products discovered from these medicinal plants have become current drug candidates [17]

1.1.2 Plant products as antimicrobial agents

Due to the increasing concerns on chemical preservatives among consumers, more attention has been given to potential antimicrobial efficacy of natural products [18-20]. These health and environmental concerns are mainly related to the carcinogenic and/or teratogenic properties of the chemical compounds and the fact that their toxic effect cumulates inside

the plant and/or soil [21, 22]. Therefore, a large number of studies have been conducted in recent years searching for the antimicrobial activity of natural products, with more attention being given to herbs and spices. Until now over 30,000 antimicrobial compounds have been isolated from more than 1340 plants with defined antimicrobial activities [23-26].

Microorganisms cause food spoilage as well as plant diseases and can develop resistance mechanism to antimicrobials faster than the development of new agricultural pesticides. Besides chemically defined pesticides, also plants are a rich source of antimicrobial compounds [27]. Among several non-synthetic chemical control options, plant extracts and their essential oils have received attention for controlling plant diseases [28]. Against post-harvest fungal diseases, numerous studies have reported the antifungal effects of plant extracts [29].

Plants extracts have not only been used against plant diseases, but humans have always searched for solvation of health problems using herbal extracts [30]. For example one of the main causes of death worldwide is infectious diseases, and due to the emergence of multidrug-resistant pathogens, there has been an increasing interest in medicinal plants as a natural alternative to synthetic drugs [31]. This increase in resistance has also been seen in fungal pathogens. For many years the only available treatment for serious fungal infections was Amphotericin B and related compounds, followed by the development of fluconazole and itraconazole in the late 1980s [32]. Unfortunately, like the multidrug-resistant bacteria, fungal pathogens are also losing their sensitivity to azole derivatives, and their resilience against them is growing, while they are still the most commonly used antifungal agents [32-37]. This matter shows the importance of finding new sources of the bioactive compounds, which can be either be used alone or combined with azole derivatives that already exist [38].

Over the past decades also fungal infections have increased. Thus a thorough search for more effective and less toxic drugs and pesticides has been made [39]. With this regard, one of the most dynamic fields of microbiology is antifungal susceptibility testing [40]. The rate of occurrence and range of infections caused by pathogenic fungi has made the in-vitro susceptibility testing even more important [41].

As it was previously described, the best source of drug discovery is the secondary metabolites produced by plants. Secondary metabolites containing sulfur atoms are an

active defense against various pathogens and pests. Interestingly, also roughly one-third of all registered pesticides contain at least one sulfur atom [42]. Therefore, it is worth to investigate sulfur-rich plant extracts such as members of the genus *Allium.*

1.2 The Genus *Allium*

"Eat leeks in March and wild garlic in May, and all the year after the physicians may play." *Traditional Welsh rhyme* [43].

1.2.1 The genus *Allium* and its phylogenetic classification

The genus *Allium* is one of the largest genera of monocots that contains more than 900 species consisting of 15 monophyletic subgenera (**Figure 1.1**) and belongs to the plant family of Alliaceae (in more recent literature Amaryllidaceae) [44, 45]. Members of the genus *Allium* occur naturally in the northern hemisphere, but eastern Mediterranean Basin as well as Southwest and Central Asia and Pakistan are believed to be their main center of diversity, where it's sunny, rather dry and moderately humid [44]. Around 50 members of this genus members are widely or locally cultivated, among which kitchen onion and shallot (*A. cepa*) as well as garlic (*A. sativum*) are quite well-known. In addition to that, people collect many wild *Allium* species not only to use as spices or vegetables but also due to their medical benefits and also in order to use as ornamentals [46]. Regarding the main characteristics of *Allium* species, member of this genus mainly have tunicated bulbs, narrow basal leaves and umbellate or head-like inflorescences [47]. The part of the plant that was used in this investigation was the bulb, and it should be considered that the shape of bulbs in genus *Allium* is very variable, from thin bulbs with a low storage capacity to very thick bulbs having a huge storage capacity [44].

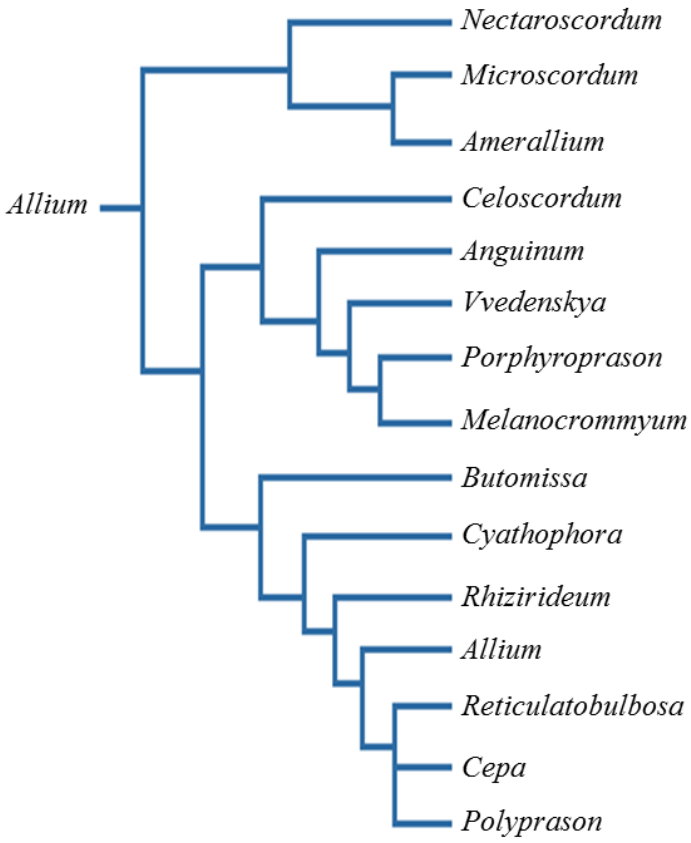


Figure 1.1 Subgenera classification of the genus *Allium* [45]

1.2.2 Pharmaceutical relevance of the genus *Allium*

The pharmaceutical benefits of garlic (*A. sativum*), as the most studied species of this genus, has been known since 1550 BC in ancient Egypt, where this plant was famous for demonstrating valuable therapeutic uses for a variety of diseases such as heart problems, headache, bites, and worms [48]. Garlic then was introduced to the Greeks and Romans as an important medicinal plant, and from that time plant has not only been used as a beneficial healing agent in the Mediterranean region but also throughout the world for its unique therapeutic effects [49]. The antibacterial properties of garlic extracts was first described by Pasteur back in 1858 [50], and epidemic diseases like typhus, cholera, diphtheria, and tuberculosis were cured using garlic even before the discovery of antibiotics, until 1944 when Cavallito and Bailey finally characterized the most potent antimicrobial agent of garlic known as allicin (**Figure 1.3**) a compound responsible for the typical smell and taste of crushed garlic [51]. In addition to its antimicrobial properties, bioactive components of garlic are also responsible for its anticancer, antioxidant, antidiabetic, hepatoprotective

effects and its potential role in preventing cardiovascular diseases [52]. It is also interesting to know that according to the "designer food project" carried out by the National Cancer Institute in the United States, garlic is positioned in the top of the pyramid of foods preventing cancer (**Figure 1.2**) [53].

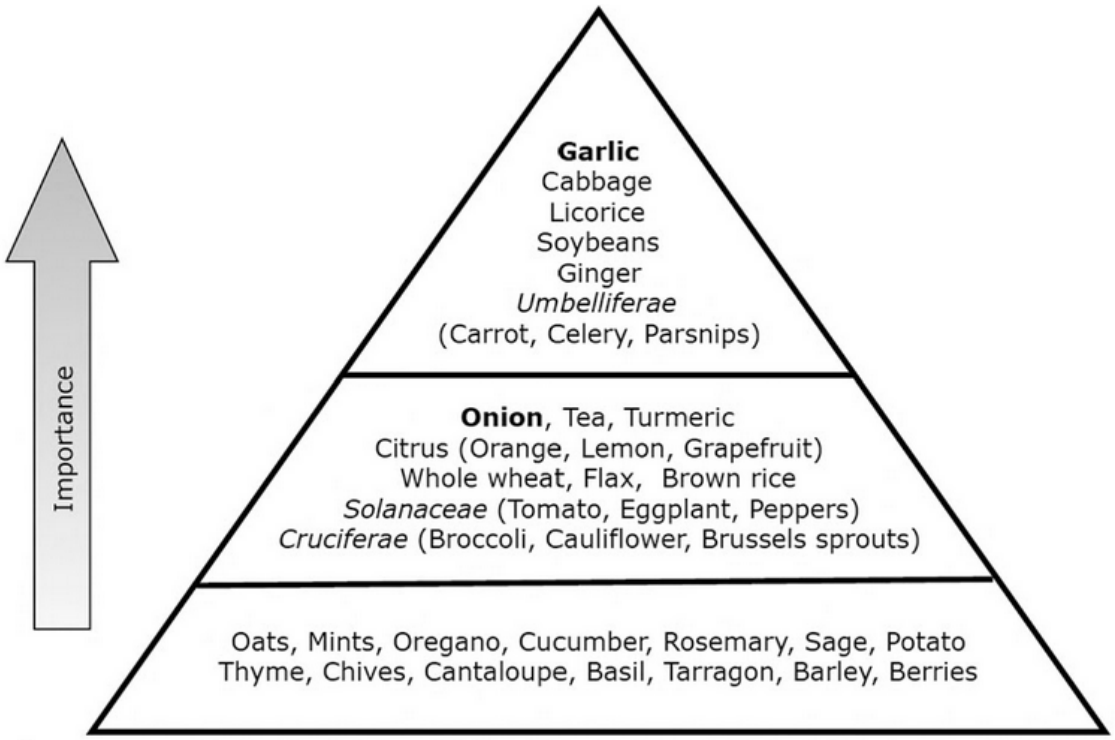


Figure 1.2 The pyramid of foods to prevent cancer [53]

Although garlic and kitchen onion is the most famous member of this group and have been very well investigated in terms of their antimicrobial activity [54], they are not the only members of genus *Allium* demonstrating antimicrobial and therapeutic effects, and allicin is also not the only potent compound causing beneficial effects. Besides garlic, other *Allium* species e.g. Persian shallot (*A. stipitatum*), leek (*A. porrum*), chive (*A. schoenoprasum*) and elephant garlic (*A. ampeloprasum*) are as well rich sources of phytonutrients like thiosulfinates, N-oxides/ N-amides, saponins and flavonoids (**Figure 1.3**) [55].

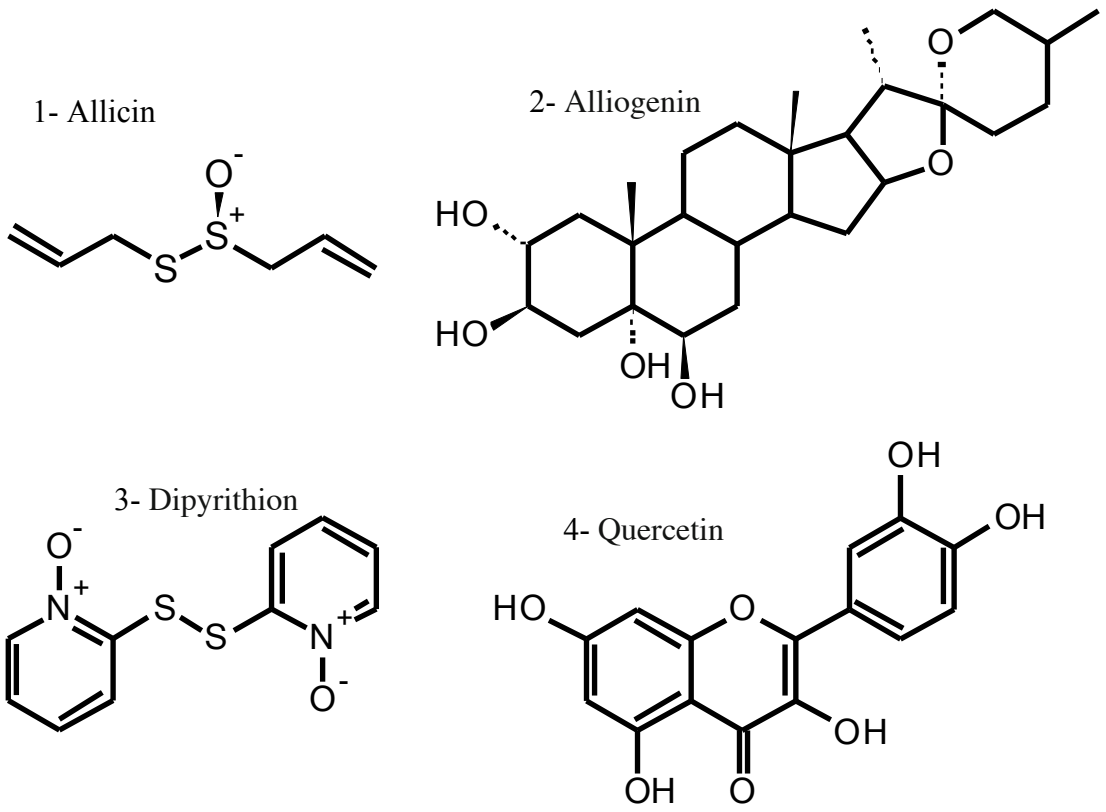


Figure 1.3 Schematic examples of antimicrobial chemical classes of natural compounds isolated from different *Allium* species, e.g., thiosulfinates (1), saponins (2), N-oxides (3) and flavonoids (4).

The importance of sulfur atom in the history of antimicrobial components has long been proved, and it is believed to be the oldest pesticide ever used by mankind [56]. Generally, sulfur-containing secondary metabolites in plants is their powerful endogenous defense against many pests and pathogens. In the past few years, volatile thiosulfinates, N-oxides, and N-amides as a sulfur-containing secondary metabolite belonging to the members of the genus *Allium* have gained much attention due to their antimicrobial effects [57]. The ability to inhibit microorganisms comes from a –S (O)–S– group, which enables these compounds to react with the SH group of cellular proteins that results in the generation of mixed disulfides [58]. On the other hand, a thiosulfinate as allicin can permeate cellular phospholipid membranes while causing no leakage, fusion or aggregation. It should be mentioned that this ability to easily overpass the cell's lipid bilayer causes a considerable influence on the cell's overall redox status, and this alternation leads to the activation of apoptosis in yeast cells [59, 60].

It should be mentioned that *Allium* species located in South West and Middle Asia exhibit a complex and diverse sulfur chemistry [61]. Therefore, lipophilic extracts of these species appear to be an outstanding source for new sulfur and aroma components. And such

diversity of sulfur compounds may also lead extracts with unrevealed biological activities like indicated by ethnopharmaceutical investigations [62]. It also can be expected that some of these extracts reveal an antimicrobial effect.

1.2.3 Alliinase reaction

Amino acid cysteine and its aliphatic derivatives, especially the S-substituted cysteine sulfoxides that are oxidized at the sulfur atom, are the most important sulfur-containing substances found inside *Allium* species. These amino acids are basically stored inside cytoplasm and are the precursors of thiosulfinates. The type of cysteine sulfoxides differs among different members of the genus *Allium*. For example, alliin (S-allyl-L-cysteine sulfoxide) can mainly be found in *A. sativum*, while *A. cepa* contains isoalliin (S-1-propenyl-L-cysteine sulfoxide). Methiin (S-methyl-L-cysteine sulfoxide) on the other hand is a universal cysteine sulfoxide which can normally be found with a high concentration in wild onions. propiin (S-propyl-L-cysteine sulfoxide) and buttin (butyl-Lcysteine sulfoxide) are also among the most important cysteine sulfoxides that play a significant part in the flavor of members of the genus *Allium* (**Figure 1.4**) [61]. Interestingly, different samples of the same species may contain variable sulfoxides, depending on factors such as postharvest storage conditions as well as genetic factors [63].

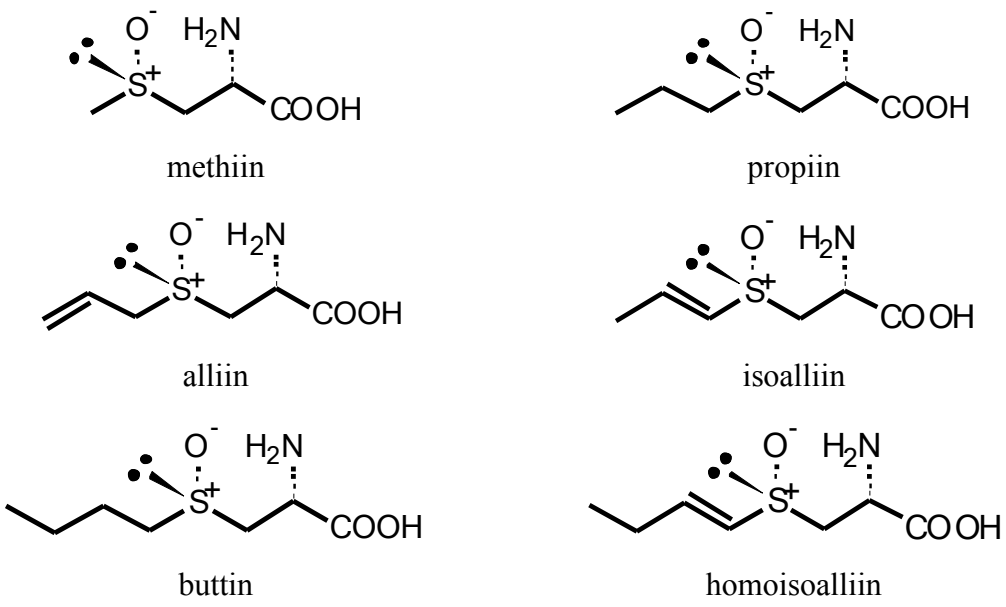


Figure 1.4 Chemical structures of the main aliphatic cysteine sulfoxides found in the members of the genus *Allium*

As cell damages, cysteine sulfoxides as cytosol-held substrates are immediately transformed into thiosulfinates, and this reaction is catalyzed by an equally-liberated vacuolar enzyme, which acts as a cysteine sulfoxide lyase, called alliinase.

Alliin alkyl-sulphenate-lyase, best known as alliinase, is one of the major protein fractions (about 10% of the total proteins [64]) found in *Allium*. This naturally-occurring diastereomer molecule enzyme was first reported in 1949 by Stoll and Seebeck [65, 66]. It is believed that this C-S lyases enzyme can be found in all species of the genus *Allium*, and plays an important role in the formation of thiosulfinate and further volatile compounds (**Figure 1.5**) [61].

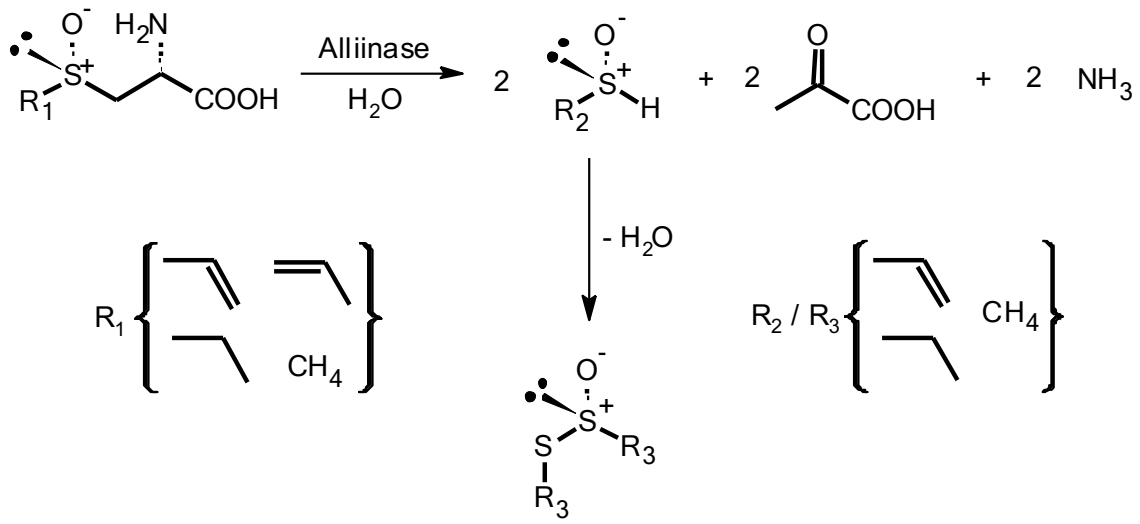
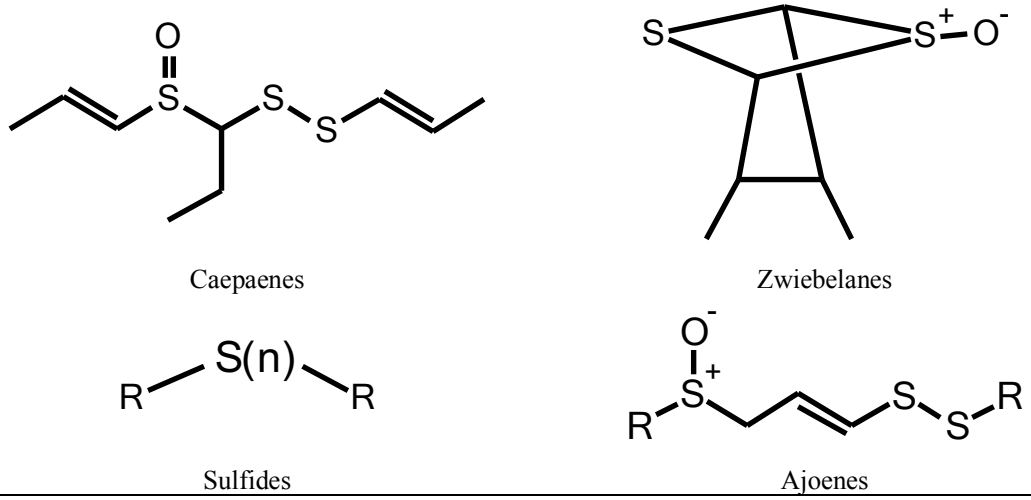


Figure 1.5 Cleavage of cysteine sulfoxides catalyzed by the enzyme alliinase

Breakage or an injury of the plant tissue enables the interaction of alliinase located mainly in vacuoles in vascular bundle sheath cells with cysteine sulfoxides gathered in the cytosol of mesophyll cells of bulbs [61, 67]. This reaction leads to the formation of pyruvic acid, ammonia, and alk(en)yl sulfenic acid. The mentioned products are quite unstable compounds, quickly converting into either corresponding thiosulfinates or the so-called primary aroma compounds [68]. Primary aroma compounds are also very reactive and unsteady resulting to their decomposition into a variety of secondary sulfur-containing compounds. It must be considered that the formation of these secondary compounds is highly dependent on the precursors (thiosulfinates formed by the alliinase reaction) as well as the reaction conditions. The most important secondary components to be mentioned here are cepaene und die zwiebelane regarding *A. cepa* and ajoene and diallylsulfide in case of *A. sativum* (**Figure 1.6**).



Caepaenes Zwiebelanes

Sulfides Ajoenes

Figure 1.6 characteristic secondary sulfur-containing compounds of *A. sativum* and *A. cepa*

*1.2.3.1*Alliinase-mediated formation of pyridyl-containing compounds in *A. stipitatum*

More than 200 species in the genus *Allium* belong to the subgenus *Melanocrommyum*. This subspecies contains a great variety of species, and its main distribution center is Southwest and Middle Asia [69]. One of the most commonly used *Allium* species belonging to this subgenus is *A. stipitatum*. This spicy vegetable and medicinal plant is mainly known as "Mu-sir" is frequently used in folk medicine for the treatment of a variety of disorders such as rheumatic and inflammatory disorders, arthritis, diarrhea, stomach pain, psoriasis, hemorrhoid inflammation and stress [62, 70, 71]. As it was mentioned before, members of the genus *Allium* mainly contain diverse biologically-active sulfur compounds, derived from various S-substituted cysteine derivatives (**Figure 1.1**). However, it was recently proved that the sulfur compounds formed in *A. stipitatum* upon tissue disruption are different, in a way that instead of thiosulfinates and sulfines, mainly pyrithione and several related sulfur-containing pyridine N-oxides are formed [72].

We still know very little about the biological properties of these sulfur-containing compounds. On 2009, O'Donnell did research on the antimicrobial and anticancer activities of these compounds, and on 2014, a study on the Anti-inflammatory and neurological activity of sulfur-containing pyridine N-oxides of *A. stipitatum* was made by Krejčová. In this study, we also tested the antifungal activity of these compounds, which proved to be very promising to cure different types of fungal infections.

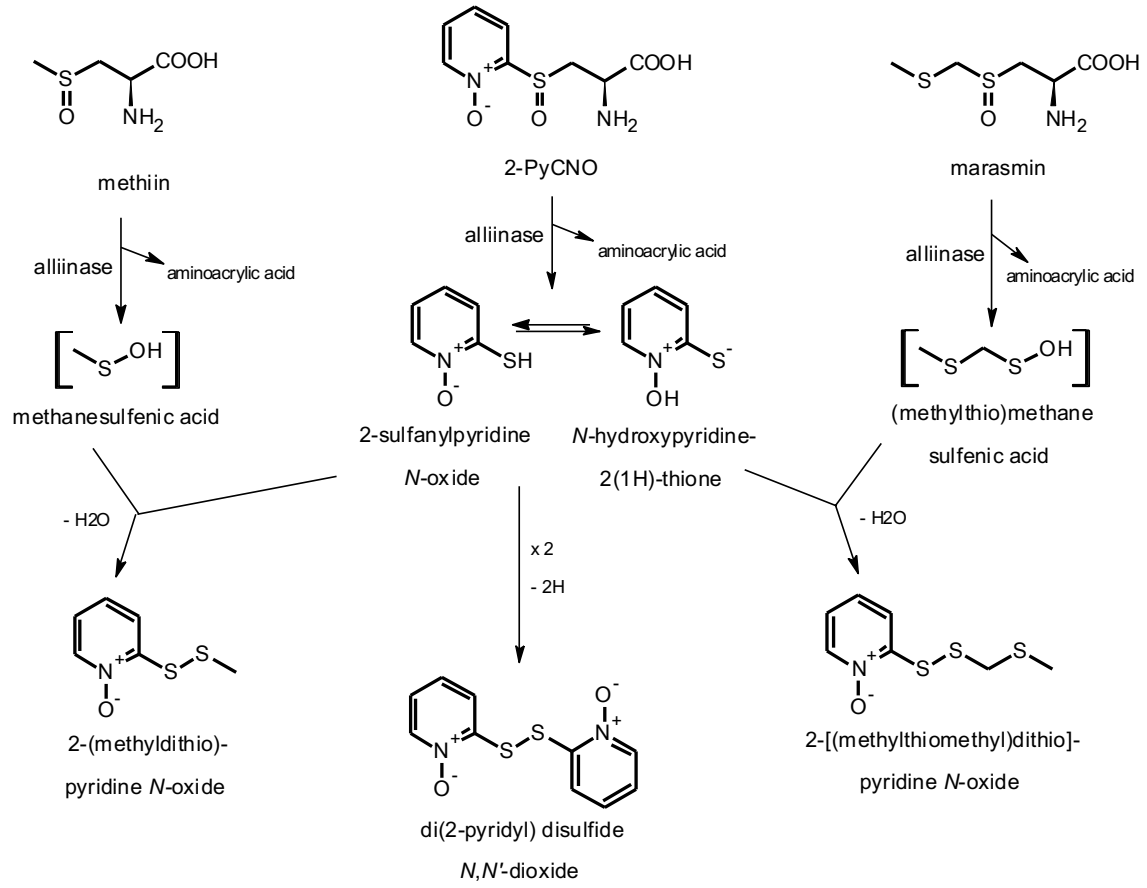


Figure 1.7 Alliinase-mediated formation of pyridyl-containing compounds in *Allium stipitatum* [72]

1.3 Fungi as plant pathogenic

It is believed that more than 70% of all plant disease is cause by over 20,000 plant pathogenic fungi, having the ability to attack different stages of the plant i.e. roots, leaves, shoots, stems, woody tissues, fruits or flowers.[73]. Among different groups of pathogens that attack plants, four very common and agriculturally important fungi were chosen to conduct the antifungal testing as follows:

1.3.1 *Aspergillus flavus*

Aspergillus flavus, filamentous fungi belonging to the phylum Ascomycota, was first described by Link back in 1809 [74]. It is an extremely common soil fungus and has a broad range of hosts, making it as one of the main problems in agriculture to be dealt with [75]. Related to the visual characteristics of this fungus, *A. flavus* is a velvety, yellow to green or brown mould, while reverse it looks goldish to red-brown, having globose to subglobose

conidia (**Figure 1.8**) [76]. As a result of producing countless airborne spores, distribution of this fungus is worldwide [77]. Although the optimum temperature for the growth of *A. flavus* is 37°C, that is its ability to grow in temperatures between 12 to 45°C, which not only enables this fungus to grow as a phytopathogen but also gives rise to its pathogenicity in humans [76].

Potentially harmful secondary metabolites produced by fungi are called mycotoxins, among which one of the most dangerous and well-known one that contaminates the crops prior to the harvest or during it, is aflatoxin, which has driven its name from "*Aspergillus flavus* toxin" [78]. The term "aflatoxin" is basically referred to a group of structurally similar toxins, among which aflatoxin B1 is the most toxic natural product ever identified, which is also believed to be a strong hepatocarcinogenic [79, 80]. On the other hand, Aspergillosis, an infection that usually affects the respiratory system [81], is also caused by *A. flavus*, normally occurring by the inhalation of the spores [82, 83]. It is also interesting to know that "the mummy's curse" or "King Tut's curse" that caused the death of a British archaeologist in Egypt, was due to the exposure to this toxin pathogen [84]. Generally, the ability of this biosafety level "2" species to not only cause infection in plants but also originating serious respiratory diseases in human, was the reason to choose *A. flavus* for this study.

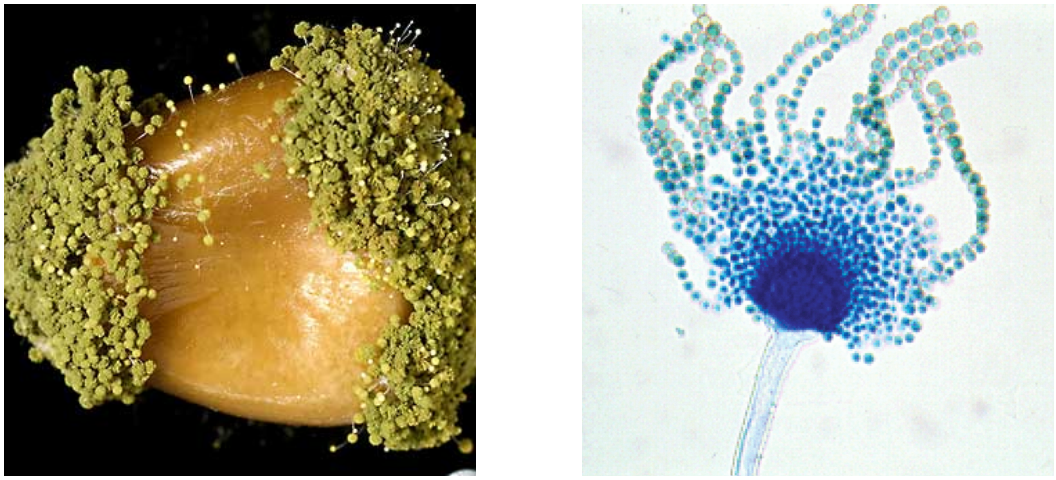


Figure 1.8 A maize kernel infected with *A. flavus* [85], microscopic features of *A. flavus* [76]

1.3.2 *Aspergillus niger*

Another worldwide distributed species belonging to the genus *Aspergillus* is *A. niger* or the black mold [86]. The characteristic that distinguishes this species from other members of the genus *Aspergillus* is the production of very dark brown to black spores (**Figure 1.9**) [87]. *A. niger* is an ubiquitous species, demonstrating the ability to survive in a high range of temperature and pH, i.e., 6-47°C and pH of 1.4-9.8 [88].

According to the US Food and Drug administration, this fungus has been categorized as GRAS (generally recognized as safe) [89], despite, it should be taken into consideration that *A. niger* is actually one of the main causes of rotting in fruits, vegetables, and other food products, which in fact leads to substantial economic loss [90]. For example, black rot of onions caused by this species generates serious losses of onion bulbs not only in the field but also during the storage [91]. Due to the production of mycotoxins, not only food producers, but also herbal medicine industries undergo huge damages from *A. niger* [89]. Due to its small and airborne conidia, the spores can be inhaled easily, leading to severe systemic or deep mycosis [92]. In addition to mycosis, this fungus is able to infect external, middle, and post-operative ear cavities, causing otomycosis, a severe ear infection [93]. Therefore *A. niger* was also chosen to undergo antifungal susceptibility tests.

Figure 1.9 Black mold, caused by *Aspergillus niger*, on white onion [94], microscopic features of *A. niger* [95]

1.3.3 *Mucor hiemalis*

The representative member of the Mucorales species, *M. hiemalis*, belongs to the phylum Zygomycota [96]. This fungus was first reported by Wehmer back in 1903 and is basically a soil-borne species, but can also be found as plant and animal parasite [97]. Regarding the phyto-pathogenicity of *M. hiemalis*, this fungus is one of the main causes of the soft rot of raspberries and strawberries. Due to the production of abundant sporangia, *M. hiemalis* causes vast infections not only in the field but also after harvest [98]. On the other hand, this species is also able to cause diseases in human too. Zygomycosis, a fungal infection that affects mainly cutaneous and sub-cutaneous tissues, is caused by the members of Zygomycota [99]. Although the optimum growth temperature of *M. hiemalis* is 32°C, there have been reports about this fungi causing zygomycosis in the immunocompromised patient [97, 99, 100]. The above-mentioned reasons, in addition to the fact that there have been very few antifungal susceptibility studies concerning this fungus, *M. hiemalis* became one of the choices regarding this investigation.

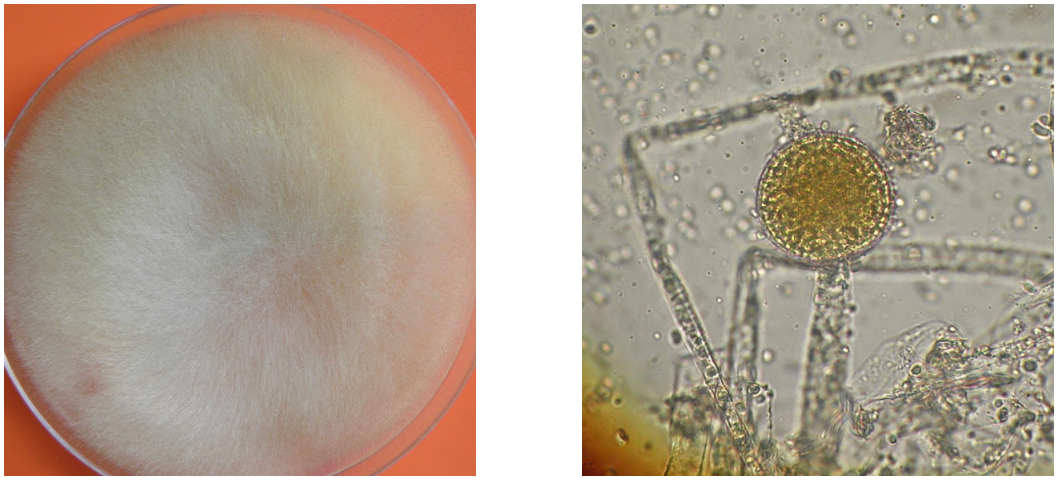


Figure 1.10 Microscopic features of *M. hiemalis* [101]

1.3.4 *Penicillium italicum*

The most economically important postharvest disease of citrus fruit is *P. italicum* or "blue mold" which was first described by Wehmer (1894). Colonies of this fungus produce blue or gray-green conidia and grow rapidly at 25°C. This fungus is not only able to infect fruits during harvest time but also causes huge losses throughout the distribution and marketing time [102]. The blue-mould of citrus fruits is the most important disease caused by *P. italicum*, which leads to soft rot of the fruit. In the advance stages, the whole surface of the fruit will become covered in a bluish green or grey-green velvety layer of conidia surrounded by a white margin [103]. The fact that *Citrus* species grow in over 100 countries worldwide and are the most widely consumed fruit by human, exhibits the importance of finding a natural and less toxic antifungal agent [104].

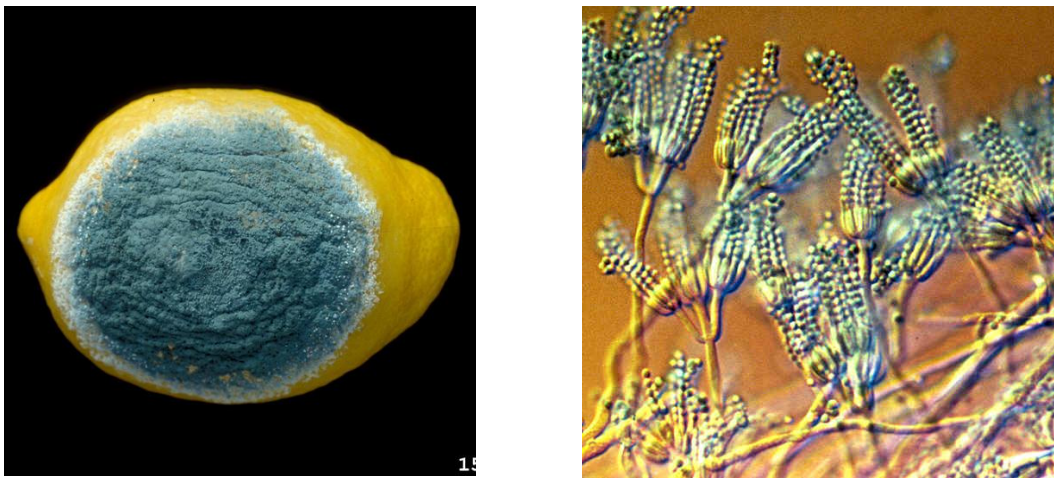


Figure 1.11 Microscopic features of *P. italicum* [105, 106]

1.4 Fungi as Human Pathogens (Dermatophyte)

The global incidence of fungal infection has been increased during the last few decades. Mycosis, which previously was considered a geographically limited disease is now a worldwide issue due to the ease of traveling. Generally, due to the increasing number of human pathogens in recent years, the recovery of nonpathogenic fungi to opportunistic invaders, and the widespread use of immunosuppressive drugs, the need for researching and developing new medicines is growing [107, 108].

From ca. 100,000 fungal species which has so far been described, around 400 are considered as a primary pathogen of man and animal [109]. Generally the infections caused by these pathogens (mycosis) can be divided into 4 categories, i.e. superficial (surface infections involving skin, hair, nail and mucous membrane), subcutaneous (mycosis of the skin, subcutaneous tissue and bones), systemic/true pathogens (inhalation of the airborne spores can cause infection in healthy immunocompetent individuals) and systemic/opportunists (fungi require a compromised host (generally patients with cancer, organ transplantation and AIDS) in order to establish infection) [108]. In this study, we chose 3 pathogens, i.e., *Basidiobolus ranarum*, *Cryptococcus neoformans* and *Epidermophyton floccosum* and belonging to subcutaneous, systemic/opportunists and superficial respectively.

1.4.1 *Basidiobolus ranarum*

B. ranarum is fungi belonging to the order Entomophthorales (phylum Zygomycota), which depending upon the environmental conditions can grow as hyphae or yeast (dimorphic) [110]. This human pathogen is most commonly found in soil, decaying organic matter, and the gastrointestinal tracts, and is believed to be part of the normal gut flora of many animals like amphibians, reptiles, fish, and bats [111]. *B. ranarum* is an opportunist meaning that it mainly causes disease in immunocompromised individuals or young children [110]. Subcutaneous zygomycosis (entomophthoromycosis) is a disease caused by *B. ranarum*, mainly occurring in tropical areas of Africa, Asia, and South America [112]. Generally this pathogen get a chance to infect human following insect bites or minor trauma to skin [113]. The symptoms of entomophthoromycosis is generally restricted to the limbs,

chest, back or buttocks. This should also be mentioned that *B. ranarum* is also capable of causing gastrointestinal infection, but this is extremely rare (**Figure 1.12**) [114].

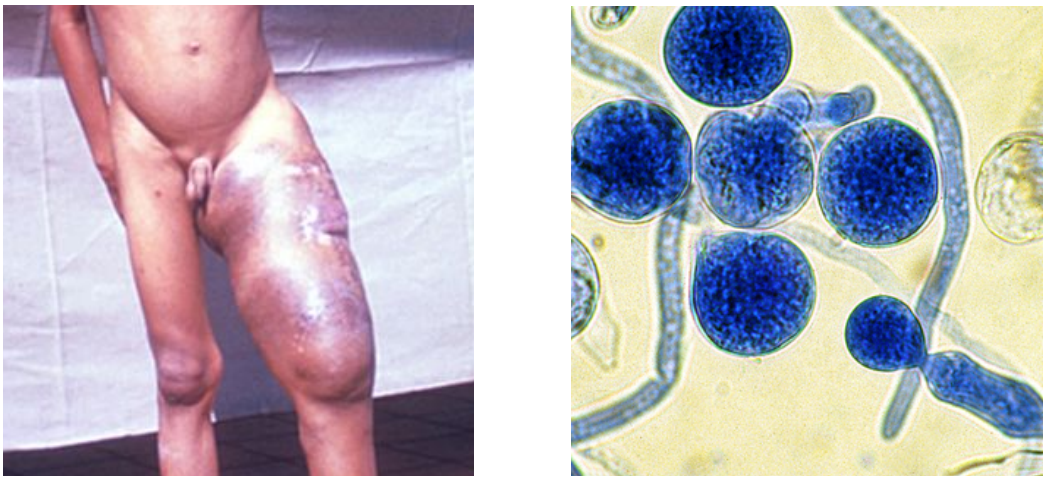


Figure 1.12 Entomophthoromycosis caused by *B. ranaurum* [115] asexual spores of *B. ranaurum* [116]

1.4.2 *Cryptococcus neoformans*

This human pathogen was first isolated from a bone infection in a young woman back in 1894, described first as a `*Saccharomyces-like*' yeast [117]. Although it has been isolated worldwide from fruits, vegetables, woods, etc. but this pathogen is mainly associated with pigeon droppings, as their chief reservoir [118]. It is a serious pathogen of mammals belonging to the fungal phylum Basidiomycota, causing a systemic disease called cryptococcosis (**Figure 1.13**), which is normally contracted by inhaling either desiccated yeast or spores [119, 120]. After contamination, immunocompetent individuals will suffer from a primary pulmonary infection, and if by any chance the host becomes immunocompromised, the organism moves to the central nervous system via lymph vessels, causing fatal meningitis [121]. This is also interesting to know that by killing over half a million individuals annually in Africa, after diarrheal disease, tuberculosis, malaria and childhood-cluster diseases, cryptococcosis stays at the 5th place regarding the infectious disease that causes deaths [122].

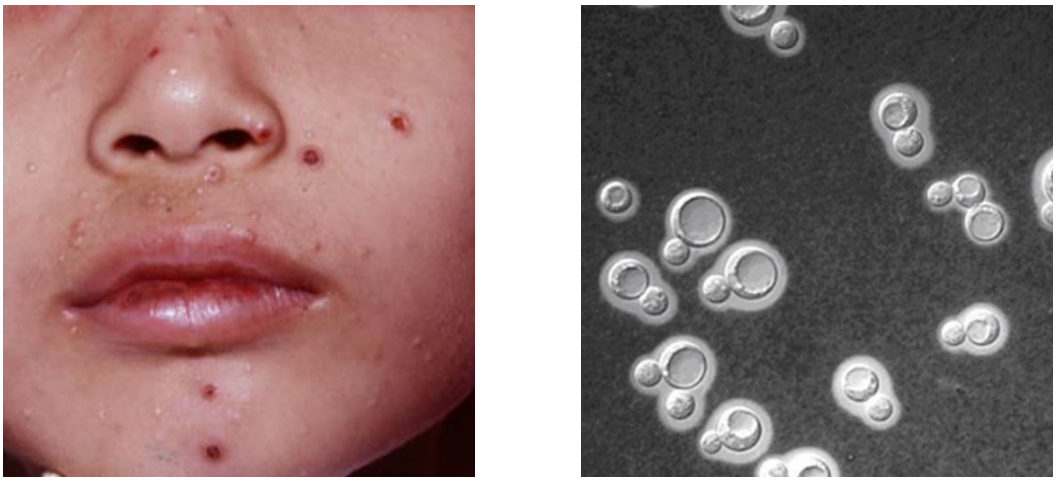


Figure 1.13 An 8-year-old HIV-infected child with cryptococcal meningitis, as well as cryptococcal skin lesions [123], *C. neoformans* yeast cells [124]

1.4.3 *Epidermophyton floccosum*

Keratinized tissue of the human body such as skin, nails, and hair can be infected by a group of fungi called Dermatophytes, which are the most common fungal infections in the world, occurring in both healthy and immunocompromised individuals [125]. This group of human pathogens is not able to penetrate viable tissue of an immunocompetent host, so their life is manly restricted to the nonliving cornified layer of the epidermis [126]. Dermatophytes mainly contain ca. 40 species divided into three main genera of, i.e., *Epidermophyton, Microsporum,* and *Trichophyton* [127]. *E. floccosum* was first described in 1870 by Carl Otto Harz. This pathogen mainly infects human, and animals and according to the body site is categorized into *Tinea corporis* (skin other than bearded area, scalp, groin, hands or feet), *Tinea cruris* (groin, perineum and perineal areas) *Tinea pedis* (feet) and *Tinea unguium* (nail) (**Figure 1.14**) [128]. *E. floccosum* and other dermatophytes can easily be transmitted through direct contact with the infected person, and there is a chance of 10 to 20% that each individual gets infected by a dermatophyte at least once in his lifetime [129]. On the other hand, recurring of dermatophytes due to their resistance to antimicrobials is one the main problems in finding a proper cure for them, which shows the importance of discovering novel antifungal agents.

Figure 1.14 *Tinea corporis* (A), *Tinea cruris* (B), *Tinea pedis* (C) and *Tinea unguium* (D) [130]

1.5 Bioassays

Bioassay (shorthand for biological assessment) is the general name given to the method or the test used for isolation of active compounds from various natural sources, and determinations of their biological activity or strength [131, 132]. It should be taken into consideration that nowadays bioassays are used in all branches of medical research and the discoveries made with them have caused great improvements in medicine [132].

1.6 Antimicrobial susceptibility testing

One of the key techniques in biological science is antimicrobial susceptibility test (AST). In order to determine to which specific product (drug or natural) a microorganism is sensitive, antimicrobial susceptibility testing can be used. A number of methods have been stablished so far in order to determine the antimicrobial susceptibility of fungi, which

generally can be categorized into two main groups: 1) dilution methods that generate minimal inhibitory concentration (MIC) which is the lowest concentration of drug that inhibits the growth of the organism, and 2) disk diffusion methods that generate a zone of inhibition [133, 134]. In this study, we used 4 different methods belonging to these two groups. A more detailed description can be found in materials and methods:

2. AIMS AND OBJECTIVES

The aim of the study is to evaluate the antifungal activity of bulb extracts of 51 *Allium* species on four plant pathogenic fungi, i.e., *Mucor hiemalis*, *Aspergillus flavus*, *Aspergillus niger,* and *Penicillium italicum*, as well as three human pathogenic fungi, i.e., *Basidiobolus ranarum*, *Cryptococcus neoformans* and *Epidermophyton floccosum*. Having discovered the most active *Allium* species, we try to isolate and characterize its bioactive compound.

2.1 Objectives

The present study is undertaken with the following objectives:

- Screening of extracts of 51 Allium species belonging to 9 different subgenera in order to evaluate their in vitro antifungal activity.
- Isolation and purification of the antifungal molecule from *Allium* samples having in vitro activity against pathogenic fungi.
- Evaluation of the antifungal potential of the isolated molecule.
- Biochemical characterization of purified molecule.

3. MATERIALS AND METHODS

3.1 List of reagents and instruments

Reagents, equipment and devices used in this investigation are listed in **Table 1**:

Reagent	Source
Potato Dextrose Agar	Sigma-aldrich
XTT sodium salt	Sigma-aldrich
Sabouraud 4% Glucose Agar	Sigma-aldrich
RPMI-1640 Medium	Sigma-aldrich
Phosphate buffered saline	Sigma-aldrich
Menadione	Sigma-aldrich
Malt extract	Sigma-aldrich
Thiazolyl Blue Tetrazolium Bromide	Sigma-aldrich
Corning® Costar® cell culture plates	Sigma-aldrich
(±)-Miconazole nitrate salt	Sigma-aldrich
Ethyl acetate	Merck, Darmstadt
Methanol	Merck, Darmstadt
Aldrithiol-2	Merck, Darmstadt
Methanol HPLC-grade	Merck, Darmstadt

Reagent	Source
Methanol HPLC-MS grade	Merck, Darmstadt
Dipyrithione	Sigma-aldrich
Equipment and devices	**Manufacturer**
SpectraMax Plus 384 Microplate Reader	Molecular Devices, Sunnyvale, CA, USA
Shimadzu SCL10Avp HPLC system	Shimadzu Suzhou Instruments, Suzhou, Jiangsu, China
pH meter Lab 850	Schott instruments, Germany
Shimadzu UV-2401 spectrometer	Shimadzu Suzhou Instruments, Suzhou, Jiangsu, China
Vortex Yellow Line TTS2	IKA, Wilmington, USA
Merck Hitachi 7000 HPLC-System	Merck KGaA, Darmstadt, Germany
Waters HPLC-System (600E System Controller, 991 Waters PDA)	Waters, Milford, USA
JEOL-ECA 500 NMR-Spektrometer	Jeol, Tokyo, Japan

Table 1 Reagents, equipment and devices used in this investigation

3.2 Plant material

Plant materials were taken from the living plant collection in IPK Gatersleben, Germany. Plant identifications were done by Dr. R. M. Fritsch of IPK. Voucher specimens were cultivated from the living Allium collections in IPK. Some plant materials were also obtained from Afghanistan (accession numbers starting with 7) in 2013, and Finland (accession numbers starting with 10) in 2014. 51 *Allium* species belonging to 9 subgenera were used in this study. A full record about the place of origin including GPS coordinates exists for all *Allium* samples[1].

[1] The origin of plants is also available via an IPK database:

(ipk-gatersleben.de/databases/genetic_resources/allium)

3.2.1 List of *Allium* samples

A full list of *Allium* sample which were used in this study are as follows:

Name	Taxon Identifier	Subgroup
***A. ampeloprasum* L.**	IPK 5728	*Allium*
***A. atroviolaceum* Boiss.**	IPK 6399	*Allium*
A. carinatum* L. subsp. *carinatum	IPK 1763	*Allium*
A. paniculatum* L. subsp. *paniculatum	IPK 0651	*Allium*
***A. pictistamineum* O. Schwarz**	IPK 5316	*Allium*
A. rotundum* L. subsp. *rotundum	IPK 2321	*Allium*
***A. rupestre* Steven**	IPK 5300	*Allium*
***A. sativum* L.**	-	*Allium*
***A. scorodoprasum* L.**	IPK 2290	*Allium*
***A. sibthorpianum* Schult. & Schult.f.**	IPK 5335	*Allium*
A. sphaerocephalon* L. subsp. *sphaerocephalon	IPK 5337	*Allium*
***A. vineale* L.**	IPK 0070	*Allium*
***A. campanulatum* S. Watson**	IPK 3061	*Amerallium*
***A. cernuum* Roth**	IPK 0068	*Amerallium*
***A. moly* L.**	IPK 0703	*Amerallium*
***A. scorzonerifolium* Desf. ex DC. var. *xericiense* Fern.**	IPK 3428	*Amerallium*
***A. victorialis* L.**	IPK 3558	*Anguinum*
***A. ramosum* L.**	IPK 5410	*Butomissa*
***A. tuberosum* Rottler ex Spreng.**	IPK 5168	*Butomissa*
***A. altaicum* Pall.**	IPK 3200	*Cepa*
***A. cepa* L. *aggregatum* group**	IPK 3216	*Cepa*
***A. cornutum* Clementi**	IPK 5193	*Cepa*
***A. maximowiczii* Regel**	IPK 2672	*Cepa*
***A. oschaninii* O. Fedtsch.**	ACC 7011	*Cepa*
***A. pskemense* B. Fedtsch.**	IPK 6178	*Cepa*
***A. schoenoprasum* L.**	ACC 100001	*Cepa*

Name	Taxon Identifier	Subgroup
***A. aflatunense* B. Fedtsch.**	IPK 1178	*Melanocrommyum*
***A. atropurpureum* Waldst. & Kit.**	IPK 1017	*Melanocrommyum*
***A. cyrilli* Ten.**	IPK 1550	*Melanocrommyum*
***A. darwasicum* Regel**	ACC 7005	*Melanocrommyum*
***A. hollandicum* R.M. Fritsch**	IPK 2802	*Melanocrommyum*
A. jesdianum* Boiss. & Buhse subsp. *jesdianum	IPK 6716	*Melanocrommyum*
***A. jesdianum* Boiss. & Buhse subsp. *angustitepalum* (Wendelbo) F.O. Khass. & R.M. Fritsch**	IPK 3671	*Melanocrommyum*
***A. karataviense* Regel**	IPK 0779	*Melanocrommyum*
***A. macleanii* Baker**	IPK 2415	*Melanocrommyum*
***A. multibulbosum* Jacq.**	IPK 5321	*Melanocrommyum*
***A. nevskianum* Vved. ex Wendelbo**	IPK 5451	*Melanocrommyum*
***A. rosenorum* R.M. Fritsch**	IPK 2530	*Melanocrommyum*
***A. stipitatum* Regel**	ACC 7002	*Melanocrommyum*
***A. obliquum* L.**	IPK 1410	*Polyprason*
***A. platyspathum* Schrenk subsp. *amblyophyllum* (Kar. & Kir.) N. Friesen**	IPK 2996	*Polyprason*
***A. talassicum* Regel**	IPK 5883	*Polyprason*
***A. jodanthum* Vved.**	IPK 5681	*Reticulatobulbosa*
***A. strictum* Schrad.**	IPK 3183	*Reticulatobulbosa*
***A. denudatum* F. Delaroche**	IPK 3470	*Rhizirideum*
***A. lusitanicum* Lam.**	IPK 5551	*Rhizirideum*
***A. nutans* L.**	IPK 5148	*Rhizirideum*
***A. rubens* Schrad. ex Willd.**	IPK 5735	*Rhizirideum*
***A. senescens* L.**	IPK 5403	*Rhizirideum*
***A. spirale* Willd.**	IPK 1642	*Rhizirideum*
***A. stellerianum* Willd.**	IPK 5738	*Rhizirideum*

Table 2 full list of *Allium* sample which were used in this study

3.3 Microorganisms

In total, seven reference strains of plant as well as human pathogenic fungi were used in this study: *Aspergillus niger* Tiegh. (IRAN 1354), *A. flavus* Link (IRAN 1426), *Penicillium italicum* Wehmer (IRAN 1049), *Mucor hiemalis* Wehmer (IRAN 911), *Cryptococcus neoformans* (San Felice) Vuill. (DSM 15466), *Basidiobolus ranarum* Eidam (DSM 957) and *Epidermophyton floccosum* (Harz) Langeron & Miloch. (DSM 10709).

3.3.1 Inoculum preparation

Preparation of the inoculum was as follows: the surface of the 7-day-old fungus plate grown over a PDA media was smoothly rubbed with 10 ml sterile distilled water containing 0.01% Tween 20 in order to collect the spores. The acquired suspension was then vortexed, centrifuged and washed 3 times using sterile distilled water. The fungal suspension was standardized to 10^6 cfu/mL.

In order to prepare the inoculum of the above-mentioned fungi, regarding the fungal type, two different methods were used. In case of *E. floccosum* which grows in the form of multicellular filaments (mold), no spores were available. Therefore, the surface of the 7-day-old fungus plate grown over potato dextrose agar was gently rubbed with sterile distilled water. The acquired suspension was then filtered using sterile gauze. Afterwards, it was vortexed centrifuged and washed 3 times using sterile distilled water. The concentration of the fungal suspension was then set to 70% transmission using a photometer at 570 nm. Preparation of the inoculum regarding *C. neoformans* and *B. ranarum* was as follows: the surface of the 7-day-old fungus plate grown over a malt extract peptone agar for *C. neoformans* and potato dextrose agar for *B. ranarum* was smoothly rubbed with 10 ml sterile distilled water containing 0.01% Tween 20 in order to collect the spores. The acquired suspension was then vortexed, centrifuged and washed 3 times using sterile distilled water. The fungal suspension was normalized to 10^7 cfu/mL.

3.4 Plant Processing

3.4.1 Plant processing for bioactivity tests

A fresh *Allium* bulb was sliced and chopped. The material was placed in 100mL of water and shaken for 45 min for the alliinase reaction to take place. The water phase was then extracted using three portions of 100 mL of EtOAc p.a. The water phase was disposed. Magnesium sulfate was then added to EtOAc as a drying agent. After filtration, the solvent was evaporated at 24˚C under reduced pressure until around 5ml residue remained. The residue was then distributed into small Eppendorf and the rest of the solvent was evaporated to an oily residue using nitrogen gas. The final oily residue was weighed and dissolved in DMSO to give the final concentration of 10mg/ml. all tests were carried out with fresh extracts and conducted using at least three replicates.

3.4.2 Plant Processing for Analysis of Volatile Sulfur Compounds

The afore mentioned method was also applicable for preparing samples used for analysis of VSCos with the only difference that the final oily residue was dissolved in methanol. Samples were analyzed immediately.

3.5 Bioassay methods

3.5.1 Broth microdilution method

The 96-well broth microdilution method for biofilm formation was originally developed for *Candida* spp. but has recently been adapted to other biofilm-forming fungal species such as *Aspergillus fumigatus* [135, 136]. This method is mainly based on the ability of metabolically active cells to reduce a tetrazolium salt (XTT) to water-soluble orange formazan compounds. The intensity of the orange color can then be determined using a microtiter-plate reader [137].

The use of small, disposable, plastic 96-well microtiter plates has made broth dilution testing very practical, low cost and reproducible. This procedure involved preparing two-fold dilutions of the antimicrobial agent in a liquid growth medium (RPMI 1640) following

its inoculation with a standardized fungal suspension. After incubation, MICs will be determined using an automated viewing device (ELISA microtiter plate reader) for inspection of each of the panel wells for growth [138].

Broth microdilution method or RPMI 1640 micro-dilution susceptibility testing method has been conducted as follows: Conidia of fungi were harvested from 7-day-old cultures on potato dextrose agar (PDA) plates by flooding the surface of the plates with 5 ml of PBS. The conidial suspension was then recovered and dispensed into a 15-ml sterile tube. Cells were then harvested by centrifugation (approximately 3,000g for 5 min at 4°C), the supernatant was then removed, and spores were washed using sterile PBS, vortexed vigorously, and centrifuged as mentioned above. The whole process was done 3 times. Later the final pellets of cells were re-suspended in approximately 20 ml of the appropriate medium (RPMI 1640 without sodium bicarbonate supplemented with L-glutamine, buffered with 165mM morpholinepropanesulfonic acid to pH 7) that has been pre-warmed to 37 °C. The spores were then counted using a hemocytometer, in order to achieve a suspension of cells at a final density of 10^7cfu/ml in RPMI 1640. From the standardized inoculum, 200 ml was pipetted into wells of the microtiter plate. The microtiter plate was covered with its original lid, sealed with parafilm, and placed in an incubator and incubated statically for 48-72h at 37°C. After biofilm formation, the microtiter plates were washed three times in sterile PBS (200–300ml per well) using a multichannel pipette to remove planktonic and/or non-adherent cells that were remained in the wells. Via a multichannel pipette, 200ml of the high working concentration of antifungal agent was added to the column 1 of each microtiter plate containing fungal biofilms, being careful not to touch or otherwise disrupt the biofilms.

100ml of RPMI 1640 was added to each well in columns 2–10. 100ml of RPMI 1640 was column 9 was the positive control (biofilm not exposed to the antifungal agent). Wells in column 10 were exposed to DMSO to observe the effect of the solvent on the results. Afterwards, 100ml of the antifungal agent from the wells of column 1 was added to the adjacent wells in column 2 (already containing100ml of medium). The contents were then mixed by gently pipetting up and down to perform a serial doubling dilution, and the pipette tips were removed. Again, 100ml of the antifungal agent from the wells of column 2 was removed and added to wells in column 3 and mixed gently. This procedure was done up to the wells of column 8, after which the final 100ml volume from the wells of column 8 were

discarded after mixing is discarded. In addition, a two-fold serial dilution of a reference antifungal compound, miconazole, was also used as the standard antifungal drug.

The plates were covered with their lids, sealed with parafilm and incubated again for 24hr at 37°C. Afterwards, they were washed once again using PBS. 20µl of MTT was added to each well, plates were then covered with aluminum foil and incubated again for 5h at 37°C. 100ml DMSO was added to each well containing MTT and shook for 1min in order to have a homogeneous mixture. Plates were then read at 570nm, using ELISA microtiter plate reader.

3.5.2 Agar microdilution method

The agar microdilution method was evolved in this project on the basis of the CLSI Broth microdilution method, combining convenience and time/cost-effectiveness of microtiter methods with the advantage of growing the fungi over the surface of the agar. The method is rapid, easy and economical providing us with easily reproducible and monitored results. Agar microdilution method can be used successfully for testing oily plant extracts. As seen in **Figure 3.1**, a twofold serial dilution of *Allium* samples and miconazole as the positive control was added to columns 1 to 8 containing the fungal spores over the surface of solid PDA media. Column 9 was our fungal growth control, and since in this case DMSO was used as the solvent, so monitoring its effect was also necessary, which was controlled in column 10. By having the test and all the controls in one microtiter plate, this method provides an easy, reliable and cheap MIC determination of oily plant extracts (**Figure 3.1**).

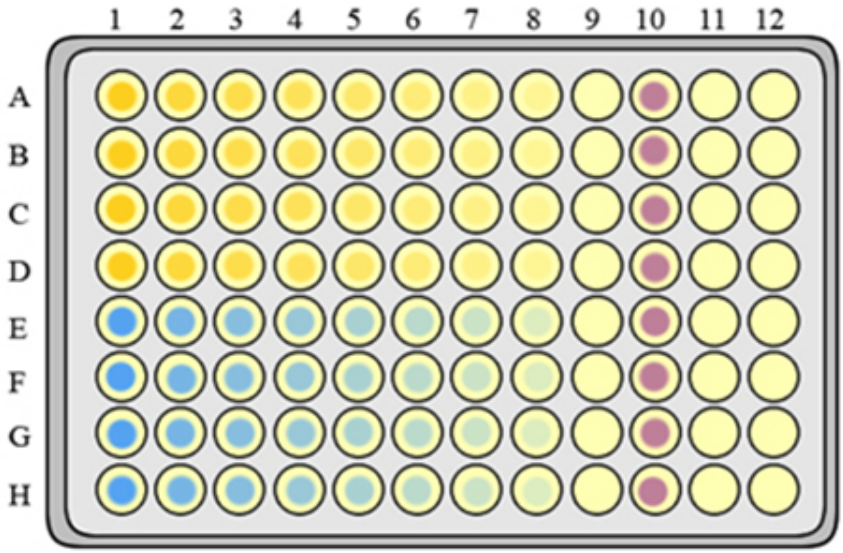


Figure 3.1 Rows A-D contain *Allium* extract starting from 10mg/mL concentration, and rows E-H contain Miconazole concentrations starting from 5mg/mL as the positive control. Column 9 controls the fungal growth, and column 10 contains DMSO as a negative control.

96-Well microtiter plates were filled with 100µl of PDA medium, and were allowed to dry out. Afterwards, 5µl of the spore inoculum of each pathogenic species was added to each well and shaken to diffuse uniformly over the PDA surface. The microtiter plate was again left to dry out for about 15min. A two-fold serial dilution of the *Allium* extract, dissolved in DMSO, was prepared in 8 small Eppendorf, with concentrations ranging from 10mg/ml to 0.08mg/ml. As positive control miconazole (purchased from Merck) was used. Dilution series of miconazole were also made in DMSO, with the concentration starting from 5mg/ml. 5µl of each concentration prepared in Eppendorf was pipetted into each well in the related column starting from 1 to 8. In order to have the control experiment in the same plate, different concentrations of the *Allium* extract and miconazole as mentioned before were pipetted into rows A-D, and E-H of every microtiter plate respectively. DMSO was used as negative control. The microtiter plate was then closed and sealed with parafilm, and stored in 24°C, with readings taken after 7 days.

Regarding the PDA micro-dilution susceptibility testing method of dermatophytes, due to the toxicity of the solvent DMSO, the *Allium* extracts were mixed directly in water. As for the positive control miconazole, since it is not water soluble, the procedure was made as follows: A two-fold serial dilution of miconazole (from 10mg/ml to 0.08mg/ml) dissolved in DMSO was made. Afterwards, a 15% solution of every Eppendorf containing a different

concentration of miconazole was made in water, resulting in the final miconazole concentration of 1.5mg/ml to 0.01mg/ml. The rest of the test was conducted similarly to plant pathogenic fungi.

3.5.3 Disk diffusion method

One of the very common methods to determine the antimicrobial sensitivity of drugs or natural products is disk diffusion method, which was first introduced in 1940 [139]. In order to perform this simple technique, a disk containing the antimicrobial agent is placed on the surface of an agar plate containing the microorganism. During the incubation, a zone of inhibition will be created due to the diffusion of the drug and the size of its zone represents the sensitivity of the microorganism toward the antimicrobial agent [140, 141] (**Figure 3.2**).

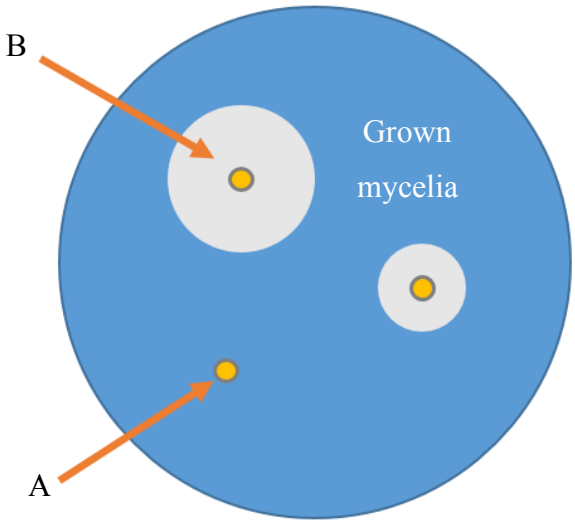


Figure 3.2 Schematic representation of disk diffusion assay for evaluation of the antifungal activity. (A) Disks of filter paper containing control solution or *Allium* extract are placed over PDA medium. (B) After incubation fungus grows throughout the dish and formation of zones of growth inhibition will be observed if the sample exerted antifungal effect.

This method has been widely used to determine the antimicrobial activity of plant extracts [142-144] due to having huge benefits. One of the main advantages of this method is its simplicity by requiring almost no special equipment. On the other hand, results can be easily interpreted, and you are flexible in regard to the disk selection for the tests. At last but definitely not least, this susceptibility method costs way less that other methods per test. This must also be taken into consideration that lack of mechanization or automation of the

test is the disadvantage of this method [138]. In addition, quantification of the amount of the antimicrobial agent diffused into the agar medium is also impossible via this method [145].

1mL of each fungal suspension was spread uniformly onto the surface of the PDA agar plates. The plates were allowed to dry for at least 30min before the disks were applied to the surface. After drying out, 6 mm diameter disks were placed on the surface of the PDA. 5µl of the extract was added to the disk. Each extract form was evaluated with 3 repetitions. The petri-dishes were sealed with parafilm and incubated at (24±2) °C, with readings taken after 72h. Zone of inhibition (ZOI) diameters for the disks were measured at the transitional point where fungi mycelial growth suddenly decreased, as determined by a reduction in density.

3.5.4 Double-dish chamber

The most common method for testing antimicrobial activity, in general, is the disk diffusion method. However, for highly lipophilic compounds as well as volatile compounds (VOCs), this method is less suitable (**Figure 3.3**). It must be pointed out that many *Allium*-related sulfur compounds are lipophilic and volatile. Therefore, two other methods were introduced in addition to the disk diffusion test: the micro-dilution test allowing the determination of the minimum inhibitory concentration (MIC) as well as the minimum effective concentration (MEC) of nearly all kinds of substances and the double-dish chamber diffusion test allowing the antimicrobial testing of volatile compounds.

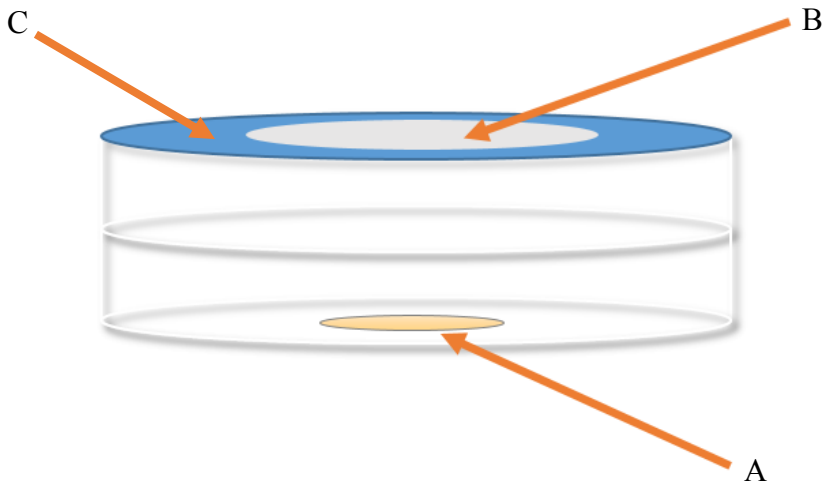


Figure 3.3 Schematic representation of double dish chamber test of volatile compounds (A) Disks of filter paper containing *Allium* extract placed inside a petri dish cap (B) a positive result (antifungal activity) is evidenced by formation of a zone of growth inhibition (C) Grown mycelia

Generally, in order to determine the effect of volatile compounds, this method can be used. 1ml of the spore inoculum containing 10^7cfu/ml prepared in sterile distilled water was added uniformly over the PDA agar plate. The plates were left to dry for at least 30min. A 2cm diameter sterile disk was then put inside the cap of the petri-dish. 50µl *Allium* extract was added over the disk. The petri-dish was then sealed with parafilm and kept upside down at 24°C for 48-72h.

3.5.5 Antimicrobial assay of different fractions

In order to evaluate the bioactivity of the HPLC micro-fractionation, drop test screening, a rapid tool to evaluate the sensitivity of the fungi to the fractions was used. Therefore, 5µL of each fraction with the concentration of 10 mg/mL was dropped onto the agar surface previously inoculated with fungal specimen. ZOI diameters was read after 72h.

3.6 Instrumental Setup for bioactivity tests

3.6.1 HPLC Separation of the Volatile Sulfur Compounds

A Waters HPLC system was used for separation. Chromatographic conditions were as follows: Isocratic methanol/water flow (A, methanol; B, water): 50% B for 10 min; 75% B

for 10 min; and 95% B for 10 min. was used with a constant flow rate of 7 ml/min through Nucleodur 100-5 C18ec column (250 × 16 mm).

3.6.2 HPLC micro-fractionation

Collection of the separated peaks following HPLC analysis was performed manually. In total, 8 fractions were in flasks and, after collection, all fractions were then extracted via EtOAc using the same procedure as mentioned above for plant processing for bioactivity tests. The content of each fraction was then weighed and suspended in DMSO for further assays evaluating the bioactivity of the fractions. A concentration of 5mg/mL was achieved.

3.6.3 HPLC-MS Analysis of the effective fractions

HPLC-ESI/MS measurements were performed on a Shimadzu SCL10Avp HPLC system containing an auto-sampler, a column oven, and a UV detector in combination with a QTrap equipped with a Turbo spray ion source. The LC ESI-MS operating conditions for the analysis of the most effective fractions (dissolved in methanol) were as follows (positive ionization mode): scan range, 30-500amu; source temperature, 300°C; ion spray voltage; flow rate, 0.25mL/min (HPLC separation). Chromatographic conditions were as follows: a methanol/water gradient with a constant flowrate of 0.25mL was used (A, methanol; B, water): 70% B for 5min; 70-5% B over 25min; and 5% B for 5min. A 100-5 C18 EC column was used for the separation.

3.6.4 Structure Elucidation

In order to achieve the structure elucidation of the effective compounds, H NMR experiments were performed on a JEOL-ECA 500 NMR-Spektrometer. HR-MS experiment was also done with an AutoSpec von Micromass. The samples were dissolved in methanol.

4. RESULTS

4.1 Selection and development of methods

One of the main challenges of this study was selection and development of screening methods. There are different types of antimicrobial susceptibility testing methods, which are standardized and can be followed to get the desired results. However, they have been developed mainly for bacteria, and in case of fungi, yeasts such as *Candida albicans*, an opportunistic human pathogen, have always been in the main focus. Regarding filamentous fungi, screening methods were also available. Yet, due to divergent behavior in growth and sporulation pattern of this group of fungi, available screening methods were barely useful.

Members of the genus *Allium* are well known for their volatile sulfur compounds. Nevertheless, available methods were mainly suitable for drug screening, as well as non-volatile plant crude extracts. Hence, in this study there was a need to stablish methods, appropriate not only for filamentous fungi, but also suitable for testing volatile compounds.

4.2 Results regarding plant pathogenic fungi

Different concentration of 51 *Allium* species was tested against 4 plant pathogenic fungi, i.e., *Mucor hiemalis*, *Aspergillus flavus*, *Aspergillus niger* and *Penicillium italicum*, using 4 different methods, i.e., broth microdilution method, Agar microdilution method, Disk

diffusion test, and double-dish chamber. In general 30 *Allium* species, that is, *A. aflatunense*, *A. ampeloprasum*, *A. atropurpureum*, *A. atroviolaceum*, *A. campanulatum, A. carinatum*, *A. cepa* var. *aggregatum*, *A. darwasicum*, *A. jodanthum*, *A. karataviense*, *A. maximowiczii*, *A. moly*, *A. multibulbosum*, *A. nevskianum*, *A. obliquum*, *A. oschaninii*, *A. paniculatum* subsp. *paniculatum*, *A. pictistamineum*, *A. pskemense*, *A. rotundum* subsp. *rotundum*, *A. rupestre*, *A. sativum*, *A. scorodoprasum*, *A. sibthorpianum*, *A. stamineum*, *A. stellerianum*, *A. stipitatum*, *A. strictum*, *A. tuberosum* and *A. vineale* demonstrated an antifungal effect either in the liquid form or volatile compounds in at least one of the above-mentioned methods. Further detailed results are as follows:

4.2.1 Broth microdilution method

As it is explained in 3.5.1 and 4.1, broth microdilution is originally made for *Candida* spp., and our aim was to extend the application to our need. Nevertheless, this method, unfortunately, did not lead to any reliable results. The main problem was due to the adherence of filamentous fungi biofilm to the bottom of the microtiter plate wells, in a way that the biofilms were mainly not stable and wiped away during washing with PBS buffer. Therefore, we decided to abandon this approach and develop the method which we will explain in the following section.

4.2.2 Agar microdilution method

This method was designed and performed due to the unsatisfying results gained by broth microdilution method. The *Allium* extract concentrations at which the MICs were measured were between 10mg/ml to 0.08mg/ml, and the MIC breakpoint of 28 tested *Allium* species, i.e. *A. aflatunense*, *A. ampeloprasum*, *A. atroviolaceum*, *A. campanulatum*, *A. carinatum*, *A. cepa* var. *aggregatum*, *A. darwasicum*, *A. jodanthum*, *A. karataviense*, *A. maximowiczii*, *A. moly*, *A. multibulbosum*, *A. nevskianum*, *A. obliquum*, *A. paniculatum* subsp. *paniculatum*, *A. pictistamineum*, *A. pskemense*, *A. rotundum* subsp. *rotundum*, *A. rupestre*, *A. sativum*, *A. scorodoprasum*, *A. sibthorpianum*, *A. stamineum*, *A. stellerianum*, *A. stipitatum*, *A. strictum*, *A. tuberosum* and *A. vineale* against plant and human pathogenic fungi were located in this range.

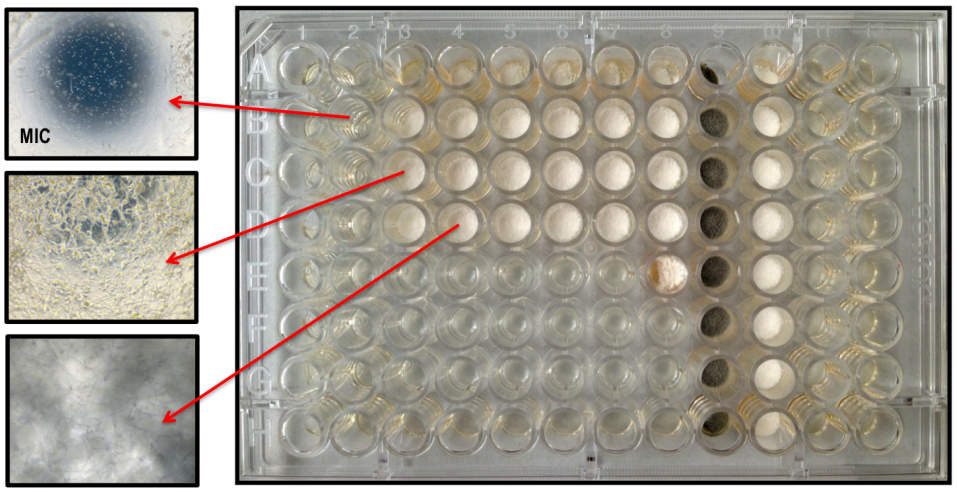


Figure 4.1 Sample plate after 7 days of incubation. Rows A-D contain *Allium* extract (*A. tuberosum*) starting from 10mg/mL concentration, and rows E-H contain miconazole concentrations starting from 5mg/mL as the positive control. Column 10 contains DMSO as a negative control. As seen in the microscopic image of well 2B, no growth can be detected, which represents the minimum inhibitory concentration (MIC) of 5.0mg/mL for extract.

The lowest minimum inhibitory concentration was presented by *A. sativum* with the MIC of 0.2mg/mL (SD=0.1) against *Aspergillus flavus*, while with the minimum inhibitory concentration of *Allium rupestre* against *M. hiemalis*, 9.9mg/mL (SD=3.6) was the least measurable breakpoint of all. More detailed results are explained in the following.

Results gained from this method regarding *M. hiemalis* showed that 20 out of 51 tested *Allium* species have an antifungal activity against this pathogen. Among the tested *Allium* samples, *A. sativum* with the average MIC of 0.5mg/mL (SD= 0.2) showed the highest antifungal activity, followed by *A. paniculatum* subsp. *paniculatum* with the average MIC of 0.6mg/mL (SD=0.3). *A. karataviense* also presented a significant effectiveness toward *M. hiemalis* with the mean MIC of 0.8mg/mL (SD=0.3). A rather high efficacy was also seen in the results gained by *A. stipitatum* and *A. pictistamineum* showing the average minimum inhibitory concentration of 1.1mg/mL (SD= 0.4) and 1.3mg/mL (SD= 0.5) respectively. The positive control, miconazole, with the average MIC of 0.58mg/mL (SD= 0.3), demonstrated the second highest concentration against *M. hiemalis*. On the other hand, *A. rupestre* showing an MIC of 9.9mg/mL (SD= 3.5) had the lowest measurable breakpoint (**Figure 4.2**, **Table 3**).

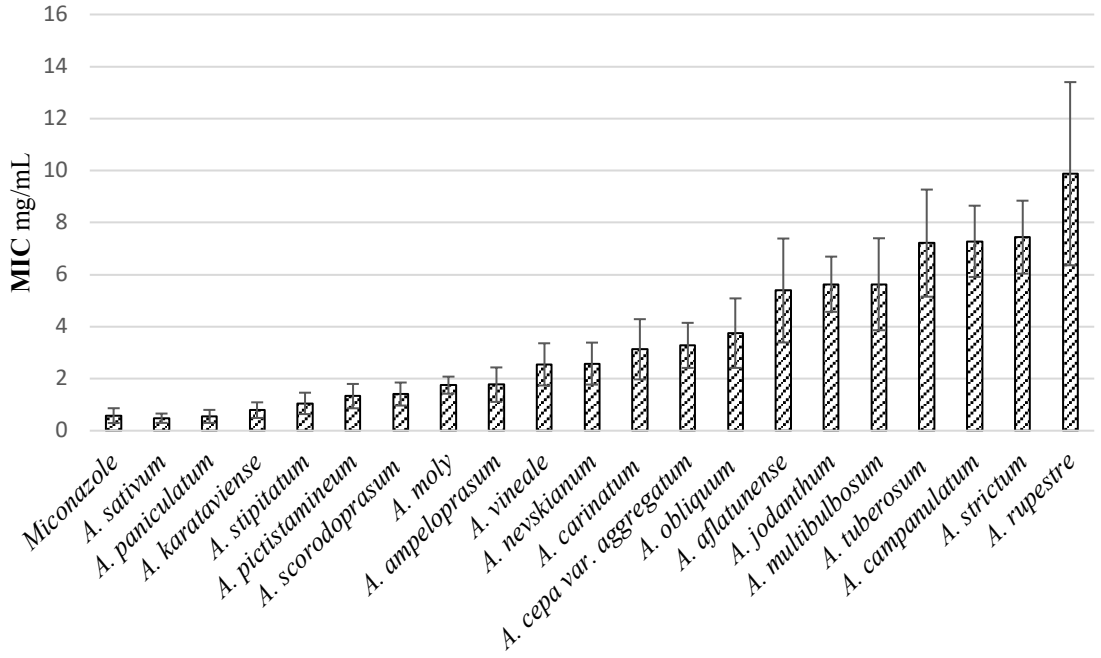


Figure 4.2 A diagram comparing MIC (mg/mL) of miconazole and the *Allium* species against *M. hiemalis*

Results obtained from the antifungal activity of the *Allium* species against *Aspergillus flavus* indicated that 21 out of 51 tested *Allium* sample demonstrated an MIC lower than 10mg/mL. The highest MIC, 0.4mg/mL (SD= 0.1), belonged to *Allium sativum* followed by *A. paniculatum* subsp. *paniculatum* and *A. aflatunense* presenting the MIC 0.6mg/mL (SD= 0.2) and 0.7mg/mL (SD= 0.3) subsequently. The minimum inhibitory concentration of *A. stipitatum* against this fungus (0.9mg/mL (SD= 0.2)) was also quite remarkable. The MIC calculated for miconazole, 0.2mg/mL (SD= 0.1), was higher than all *Allium* samples tested against *Aspergillus flavus* (**Figure 4.3**, **Table 3**).

The next pathogen, *A. niger*, showed the highest susceptibility towards *A. sativum*, with the minimum inhibitory concentration of 0.2mg/mL (SD= 0.1). The effectiveness of *A. sativum* was higher than the MIC of miconazole with 0.2mg/mL (SD= 0.1). In general, 24 out of 51 *Allium* species had a detectable effect against *A. niger.* Other effective species are *A. paniculatum* subsp. *paniculatum*, *A. karataviense* and *A ampeloprasum* with an average MIC of 0.6mg/mL (SD= 0.2), 0,7mg/mL (SD= 0.2) and 0.7mg/mL (SD= 0.3) respectively (**Figure 4.4**, **Table 3**).

In contrary to *A. niger*, which proved to be the most susceptible fungus towards *Allium* species, only 14 out of 51 tested *Allium* samples against *P. italicum* showed to have an

MICs under 10mg/mL. this fact makes this fungus the most resistant one. The antifungal susceptibility testing regarding *P. italicum* indicated again that this fungus is most susceptible towards *A. sativum* presenting the MIC of 0.2mg/mL (SD= 0.1). The minimum inhibitory concentration acquired by *A. stipitatum* was comparable to the results gain by *A. sativum* with the MIC of 0.3mg/mL (SD= 0.1). *A. paniculatum* subsp. *paniculatum* (MIC: 0.7mg/mL, SD= 0.3) also exhibited a high antifungal activity against *P. italicum*. The susceptibility toward miconazole could not be clearly determined (<0.08 mg/mL) since the breakpoint was less than the microdilution endpoint (10-0.08mg/mL) (**Figure 4.5**; **Table 3**).

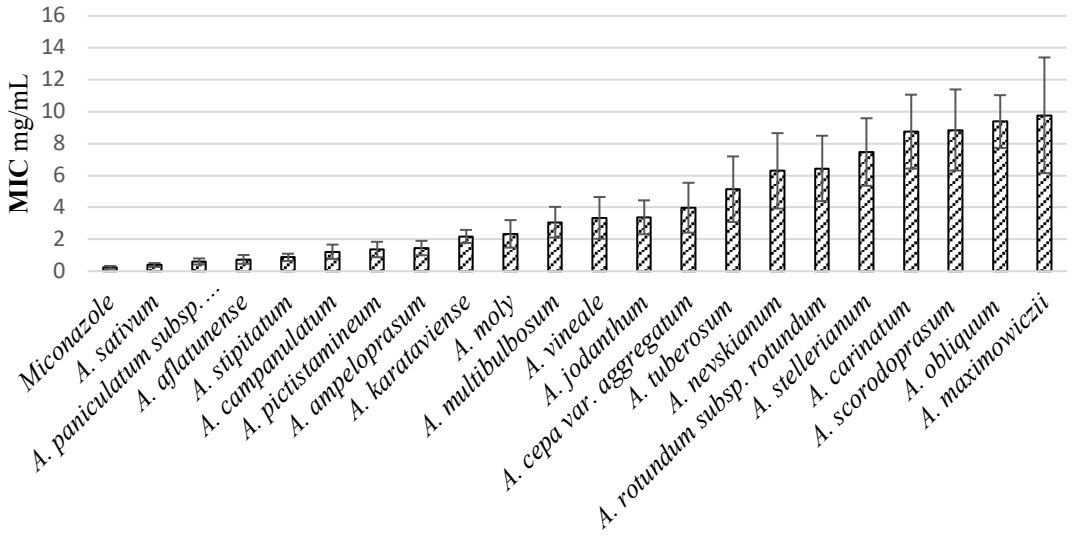


Figure 4.3 A diagram comparing MIC (mg/mL) of miconazole and the *Allium* species against *A. flavus*

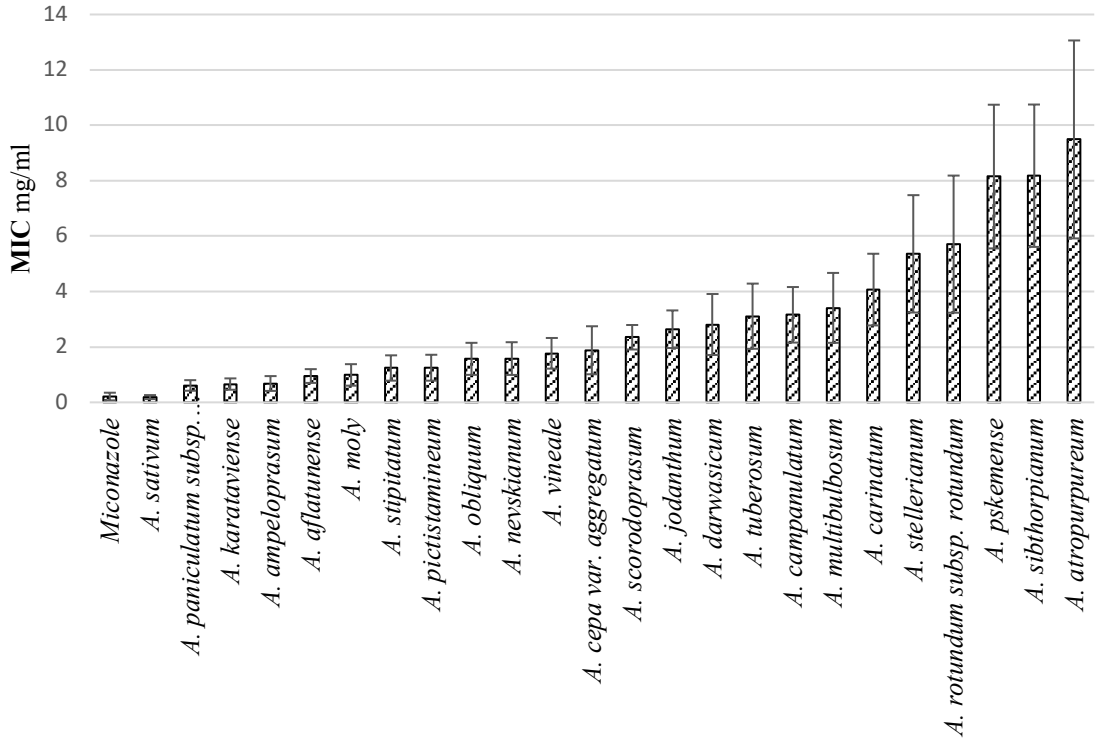


Figure 4.4 A diagram comparing MIC (mg/mL) of miconazole and the *Allium* species against *A. niger*

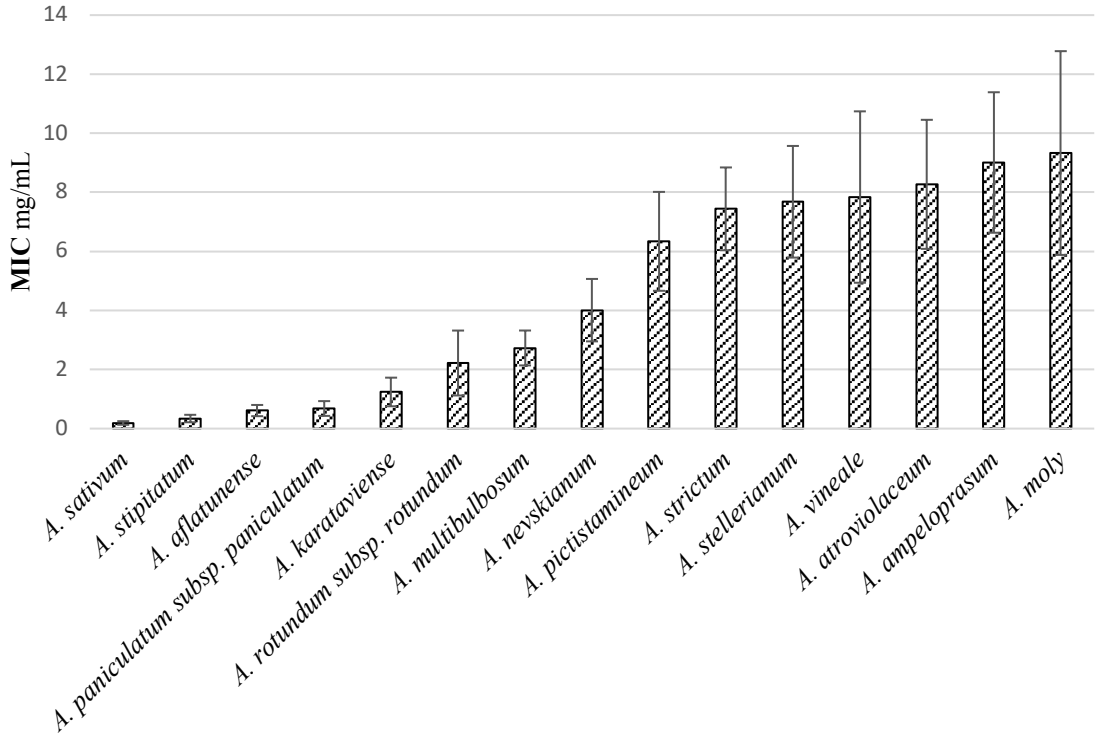


Figure 4.5 A diagram comparing MIC (mg/mL) of miconazole and the *Allium* species against *P. italicum*

Name	Subgenus	MIC [mg/mL]	Standard deviation [mg/mL]
Mucor hiemalis			
Miconazole	-	0.6	0.3
A. sativum	*Allium*	0.5	0.2
A. paniculatum subsp. *paniculatum*	*Allium*	0.6	0.3
A. karataviense	*Melanocrommyum*	0.8	0.3
A. stipitatum	*Melanocrommyum*	1.1	0.4
A. pictistamineum	*Allium*	1.3	0.5
A. scorodoprasum	*Allium*	1.4	0.4
A. moly	*Amerallium*	1.8	0.3
A. ampeloprasum	*Allium*	1.8	0.7
A. vineale	*Allium*	2.6	0.8
A. nevskianum	*Melanocrommyum*	2.6	0.8
A. carinatum	*Allium*	3.1	1.2
A. cepa var. *aggregatum*	*Cepa*	3.3	0.9
A. obliquum	*Polyprason*	3.8	1.3
A. aflatunense	*Melanocrommyum*	5.4	2.0
A. jodanthum	*Reticulatobulbosa*	5.6	1.1
A. multibulbosum	*Melanocrommyum*	5.6	1.8
A. tuberosum	*Butomissa*	7.2	2.1
A. campanulatum	*Amerallium*	7.3	1.3
A. strictum	*Reticulatobulbosa*	7.4	1.4
A. rupestre	*Allium*	9.9	3.6
Aspergillus flavus			
Miconazole		0.2	0.1
A. sativum	*Allium*	0.4	0.1
A. paniculatum subsp. *paniculatum*	*Allium*	0.6	0.2
A. aflatunense	*Melanocrommyum*	0.7	0.3
A. stipitatum	*Melanocrommyum*	0.8	0.2
A. campanulatum	*Amerallium*	1.2	0.4
A. pictistamineum	*Allium*	1.4	0.5

Name	**Subgenus**	**MIC [mg/mL]**	**Standard deviation [mg/mL]**
A. ampeloprasum	*Allium*	1.5	0.5
A. karataviense	*Melanocrommyum*	2.2	0.4
A. moly	*Amerallium*	2.3	0.9
A. multibulbosum	*Melanocrommyum*	3.1	1.0
A. vineale	*Allium*	3.3	1.3
A. jodanthum	*Reticulatobulbosa*	3.4	1.1
A. cepa var. *aggregatum*	*Cepa*	4.0	1.6
A. tuberosum	*Butomissa*	5.1	2.1
A. nevskianum	*Melanocrommyum*	6.3	2.4
A. rotundum subsp. *rotundum*	*Allium*	6.4	2.1
A. stellerianum	*Rhizirideum*	7.5	2.1
A. carinatum	*Allium*	8.8	2.3
A. scorodoprasum	*Allium*	8.9	2.5
A. obliquum	*Polyprason*	9.4	1.7
A. maximowiczii	*Cepa*	9.8	3.6
Aspergillus niger			
Miconazole		0.2	0.1
A. sativum	*Allium*	0.2	0.1
A. paniculatum subsp. *paniculatum*	*Allium*	0.6	0.2
A. karataviense	*Melanocrommyum*	0.7	0.2
A. ampeloprasum	*Allium*	0.7	0.3
A. aflatunense	*Melanocrommyum*	1.0	0.2
A. moly	*Amerallium*	1.0	0.4
A. stipitatum	*Melanocrommyum*	1.2	0.5
A. pictistamineum	*Allium*	1.3	0.5
A. obliquum	*Polyprason*	1.6	0.6
A. nevskianum	*Melanocrommyum*	1.6	0.6
A. vineale	*Allium*	1.8	0.6
A. cepa var. *aggregatum*	*Cepa*	1.9	0.9
A. scorodoprasum	*Allium*	2.4	0.4

Name	Subgenus	MIC [mg/mL]	Standard deviation [mg/mL]
A. jodanthum	*Reticulatobulbosa*	2.6	0.7
A. darwasicum	*Melanocrommyum*	2.8	1.1
A. tuberosum	*Butomissa*	3.1	1.2
A. campanulatum	*Amerallium*	3.2	1.0
A. multibulbosum	*Melanocrommyum*	3.4	1.3
A. carinatum	*Allium*	4.1	1.3
A. stellerianum	*Rhizirideum*	5.4	2.1
A. rotundum subsp. *rotundum*	*Allium*	5.7	2.5
A. pskemense	*Cepa*	8.2	2.6
A. sibthorpianum	*Allium*	8.2	2.6
A. atropurpureum	*Melanocrommyum*	9.5	3.6
Penicillium italicum			
A. sativum	*Allium*	0.2	0.1
A. stipitatum	*Melanocrommyum*	0.3	0.1
A. aflatunense	*Melanocrommyum*	0.6	0.2
A. paniculatum subsp. *paniculatum*	*Allium*	0.7	0.3
A. karataviense	*Melanocrommyum*	1.3	0.5
A. rotundum subsp. *rotundum*	*Allium*	2.2	1.1
A. multibulbosum	*Melanocrommyum*	2.7	0.6
A. nevskianum	*Melanocrommyum*	4.0	1.1
A. pictistamineum	*Allium*	6.3	1.7
A. strictum	*Reticulatobulbosa*	7.4	1.4
A. stellerianum	*Rhizirideum*	7.7	1.9
A. vineale	*Allium*	7.9	2.9
A. atroviolaceum	*Allium*	8.3	2.2
A. ampeloprasum	*Allium*	9.0	2.4
A. moly	*Amerallium*	9.3	3.4

Table 3 Detailed results of the minimum inhibitory concentrations and their standard deviations regarding *Mucor hiemalis*, *Aspergillus flavus*, *A. niger* and *Penicillium italicum*.

4.2.3 Disk diffusion test

Results regarding the disk diffusion method indicated that out of 51 tested *Allium* sample, 16 species, i.e., *A. aflatunense, A. ampeloprasum, A. campanulatum, A. cepa var. aggregatum, A. karataviense, A. moly, A. multibulbosum, A. nevskianum, A. paniculatum subsp. paniculatum , A pictistamineum, A. sativum, A. sibthorpianum, A. stellerianum, A. stipitatum, A. tuberosum and A. vineale* demonstrated a positive results toward one or all of the tested pathogens, that is *Mucor hiemalis*, *Aspergillus flavus*, *A. niger* and *Penicillium italicum* (**Table 4**).

11 out of 51 tested *Allium* samples showed a positive result toward *M. hiemalis*. As seen in **Figure 4.6**, *M. hiemalis* was mostly susceptible to *A. karataviense* with the average inhibition zone of 21mm (SD= 1) in diameter. *A. stipitatum* and *A. sativum* with the average ZOI of 16mm (SD= 1) and 15mm (SD= 2) respectively, caused smaller inhibition zones than *A. karataviense. A. aflatunense* and *A. pictistamineum* showing a ZOI of 15mm (SD= 1) and 14mm (SD=1) subsequently were almost as effective as *A. stipitatum* and *A. sativum*. Miconazole with the average inhibition zone of 9mm (SD=0), generated narrower inhibition zones than other *Allium* samples except for *A. multibulbosum* and *A. campanulatum* (**Figure 4.6**).

Aspergillus flavus was also susceptible towards 11 sample out of 51 tested species. This pathogen shows highest susceptibility towards *A. stipitatum* with a ZOI of 26mm (SD= 2), followed by *A. aflatunense* and *A sativum* with average zones of inhibition of 25mm (SD=3) and 20mm (SD= 2). *A. karataviense* showing the ZOI of 19mm (SD=1) was quite comparable with *A. sativum.* The ZOI of 23 indicates the high degree of sensitivity of *Aspergillus flavus* to a miconazole (**Figure 4.7**).

Results gained by *A. niger* showed that this pathogen is the most susceptible to all the testes fungi toward *Allium* samples with 16 positive results out of 51. *A. sativum* causing a ZOI of 34mm (SD= 3) proved to be the most effective *Allium* sample. Zone of inhibition caused by the following three *Allium* species, i.e., *A. aflatunense*, *A. karataviense* and *A. stipitatum* were more or less the same with the average ZOI of 21mm (SD= 1), 20mm (SD= 1) and 20mm (SD= 0) subsequently. Zone of inhibition generated by miconazole was 18mm (SD= 1) which proved not even to be as strong as the *A. paniculatum* subsp. *paniculatum* demonstrating a ZOI of 20mm (SD= 2) (**Figure 4.8**).

Penicillium italicum demonstrated significant sensitivity toward *A. stipitatum* with an average zone of inhibition of 31mm (SD= 3) in diameter, following by *A. sativum* and *A. aflatunense* with the average ZOI of 29mm (SD= 2) and 27mm (SD=1) subsequently. This pathogenic fungus was the most susceptible to all tested pathogens towards miconazole with the average zone of inhibition of 30.2mm (SD= 3) in diameter. In general, only 9 *Allium* samples were able to create a ZOI toward *P italicum*, which makes this pathogen the most resistant pathogenic fungi in this test (**Figure 4.9**).

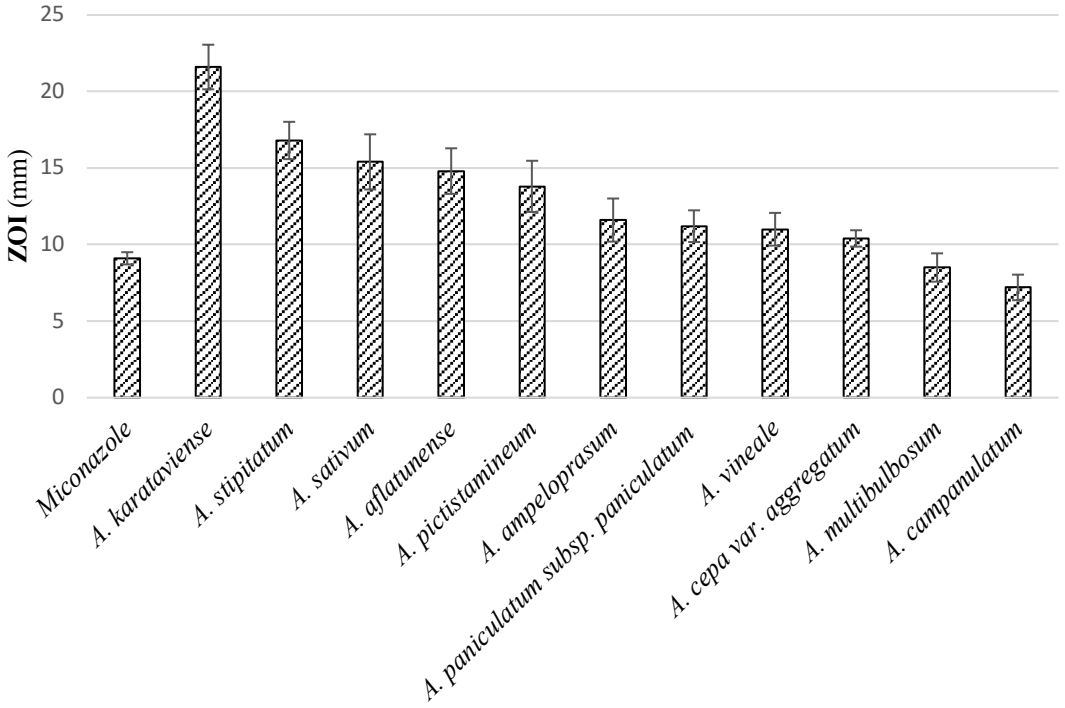


Figure 4.6 A diagram comparing ZOI (mm) of miconazole and the *Allium* species against *Mucor hiemalis*

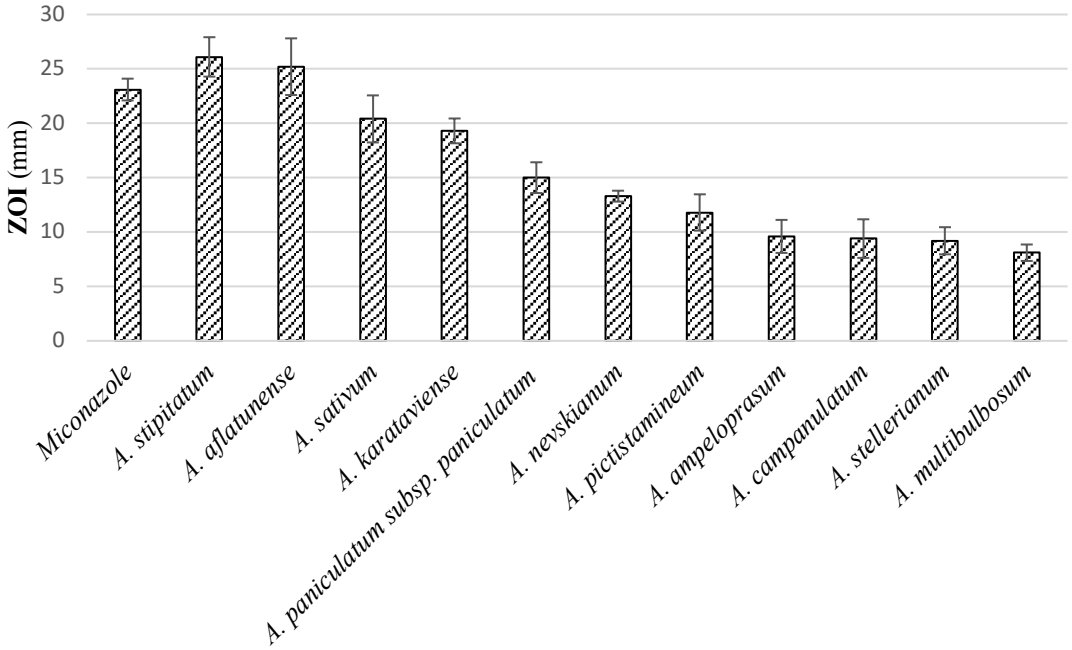


Figure 4.7 A diagram comparing ZOI (mm) of miconazole and the *Allium* species against *Aspergillus flavus*

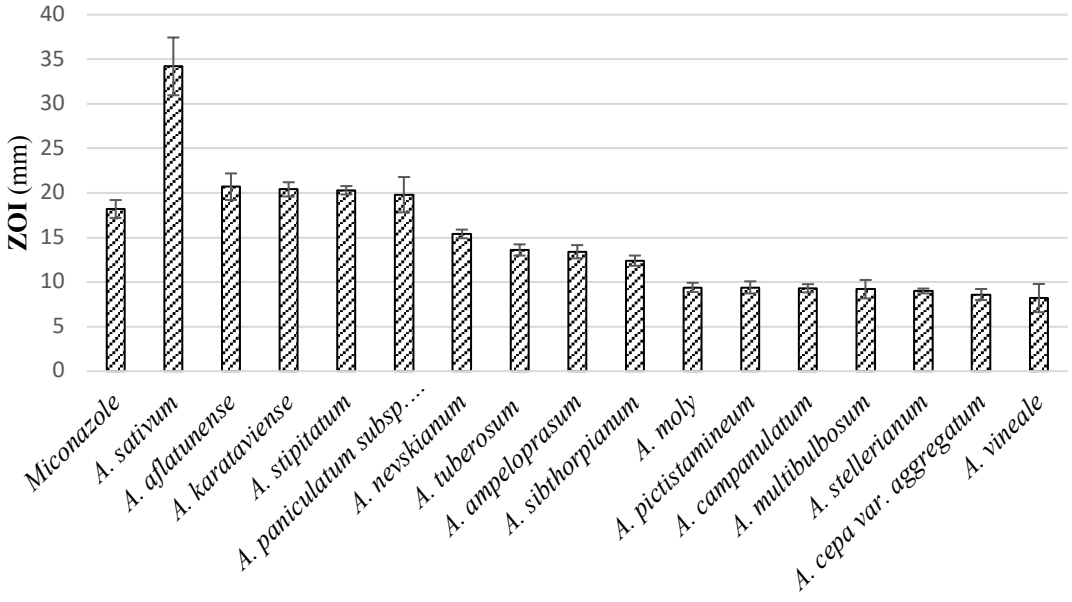


Figure 4.8 A diagram comparing ZOI (mm) of miconazole and the *Allium* species against *Aspergillus niger*

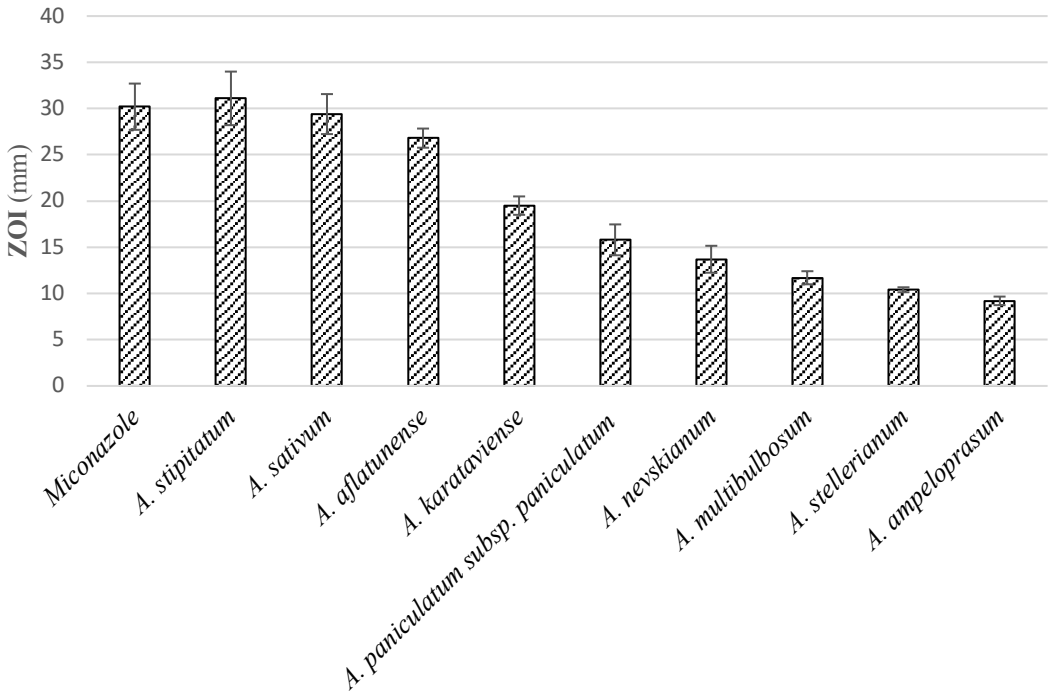


Figure 4.9 A diagram comparing ZOI (mm) of miconazole and the *Allium* species against *Penicillium italicum*

Name	Subgenus	ZOI (mm)	Standard deviation (mm)
Mucor hiemalis			
Miconazole		9	0
A. karataviense	*Melanocrommyum*	22	1
A. stipitatum	*Melanocrommyum*	17	1
A. sativum	*Allium*	15	2
A. aflatunense	*Melanocrommyum*	15	1
A. pictistamineum	*Allium*	14	2
A. ampeloprasum	*Allium*	12	1
A. paniculatum subsp. *paniculatum*	*Allium*	11	1
A. vineale	*Allium*	11	1
A. cepa var. *aggregatum*	*Cepa*	10	1
A. multibulbosum	*Melanocrommyum*	9	1
A. campanulatum	*Amerallium*	7	1
Aspergillus flavus			

Name	**Subgenus**	**ZOI (mm)**	**Standard deviation (mm)**
Miconazole		23	1
A. stipitatum	*Melanocrommyum*	26	2
A. aflatunense	*Melanocrommyum*	25	3
A. sativum	*Allium*	20	2
A. karataviense	*Melanocrommyum*	19	1
A. paniculatum subsp. *paniculatum*	*Allium*	15	1
A. nevskianum	*Melanocrommyum*	13	1
A. pictistamineum	*Allium*	12	2
A. ampeloprasum	*Allium*	10	2
A. campanulatum	*Amerallium*	9	2
A. stellerianum	*Rhizirideum*	9	1
A. multibulbosum	*Melanocrommyum*	8	1
Aspergillus niger			
Miconazole		18	1
A. sativum	*Allium*	34	3
A. aflatunense	*Melanocrommyum*	21	1
A. karataviense	*Melanocrommyum*	20	1
A. stipitatum	*Melanocrommyum*	20	0
A. paniculatum subsp. *paniculatum*	*Allium*	20	2
A. nevskianum	*Melanocrommyum*	15	0
A. tuberosum	*Butomissa*	14	1
A. ampeloprasum	*Allium*	13	1
A. sibthorpianum	*Allium*	12	1
A. moly	*Amerallium*	9	1
A. pictistamineum	*Allium*	9	1
A. campanulatum	*Amerallium*	9	0
A. multibulbosum	*Melanocrommyum*	9	1
A. stellerianum	*Rhizirideum*	9	0
A. cepa var. *aggregatum*	*Cepa*	9	1
A. vineale	*Allium*	8	2

Name	Subgenus	ZOI (mm)	Standard deviation (mm)
Penicillium italicum			
Miconazole		30	3
A. stipitatum	*Melanocrommyum*	31	3
A. sativum	*Allium*	29	2
A. aflatunense	*Melanocrommyum*	27	1
A. karataviense	*Melanocrommyum*	20	1
A. paniculatum subsp. *paniculatum*	*Allium*	16	2
A. nevskianum	*Melanocrommyum*	14	1
A. multibulbosum	*Melanocrommyum*	12	1
A. stellerianum	*Rhizirideum*	10	0
A. ampeloprasum	*Allium*	9	0

Table 4 Detailed results of the zone of inhibitions and their standard deviations regarding *Mucor hiemalis*, *Aspergillus flavus*, *A. niger,* and *Penicillium italicum*.

4.2.4 Double-dish chamber

In contrast to the normal disk diffusion method, the disk is not in direct contact with the agar plate (**Figure 4.10**). This means that only volatile compound can cause a ZOI.

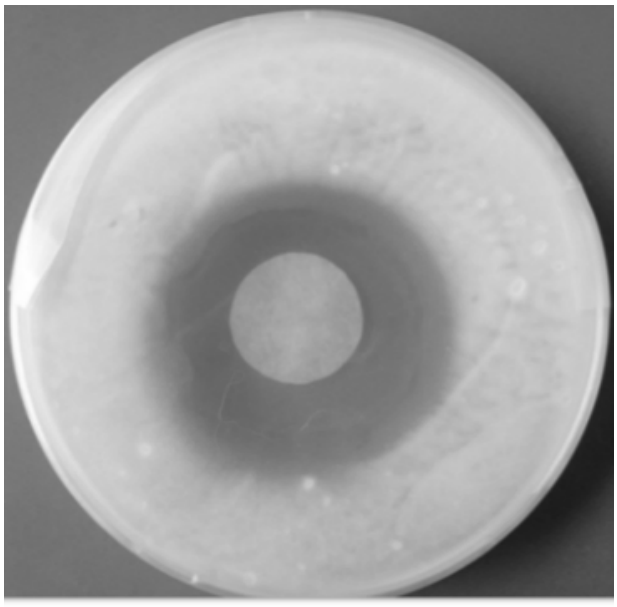


Figure 4.10 Test of volatile compounds by the double-dish chamber method (sample from *A. sativum*). The photo shows the double dish chamber after 72h of incubation from the top.

Among all examined *Allium* samples, only 10 species belonging to 6 subgenera displayed positive results. As shown in **Table 1**, the largest ZOI was caused by *A. sativum* against *P. italicum* and *A. niger* with diameters of 66-68mm and 63-66mm, respectively. *A. paniculatum* subsp. *paniculatum* also showed significant inhibition specifically towards *A. niger* with a ZOI of 50-56mm. Only, in this case, sporulation on the whole agar plate has been completely inhibited due to the effect of volatile compounds. In some other tests, e.g., *A. ampeloprasum* against *P. italicum*, only inhibition of sporulation was observed but no ZOI. The obtained data suggested that *A. niger* showed the highest susceptibility towards *Allium* volatile compounds, while *P. italicum* appeared to be the most resistant pathogen.

Name	***A. niger***	***A. flavus***	***P. italicum***	***M. hiemalis***
A. obliquum	30-32	-	-	-
A. oschaninii	18-20 *	-	-	27-30 *
A. paniculatum subsp. paniculatum	50-56 **	40-44	36-40	24-30
A. rupestre	30-34	24-26	-	-
A. scorodoprasum	20-23	20-22	-	-

Name	*A. niger*	*A. flavus*	*P. italicum*	*M. hiemalis*
A. tuberosum	50-60	25-28	-	29-31
A. ampeloprasum	47-49	38-40	15-18 *	27-30
A. cepa var. aggregatum	37-39	19-21 *	-	17-19
A. karataviense	3-5	6-9		
A. moly	18-21	24-26 *	-	-
A. sativum	63-66	42-45	66-68	30-32

Table 5 Zone of inhibition (ZOI) diameter in mm of the *Allium* species antifungal activity regarding the volatile compounds against the pathogenic fungi. (*) In some tests, the inhibition zone contained sparse fungal growth instead of complete inhibition. However no sporulation occurred in this zone. (**) In addition to the zone of inhibition, no sporulation was seen in the whole plate.

4.3 Results regarding human pathogenic fungi

Different concentration of 7 *Allium* species, that is, *A. karataviense, A. moly, A. nevskianum, A. paniculatum* subsp. *paniculatum, A. pictistamineum, A. sativum* and *A. stipitatum* which proved to have a high antifungal activity when tested against plant pathogenic fungi were tested against 3 human pathogenic fungi, i.e., *Basidiobolus ranarum*, *Cryptococcus neoformans* and *Epidermophyton floccosum*, using 3 different methods, that is, agar microdilution method, disk diffusion test, and double-dish chamber. In general, all *Allium* species demonstrated an antifungal effect either in the liquid form or volatile compounds in at least one of the above-mentioned methods. Further detailed results are as follows:

4.3.1 Agar microdilution method

Results gained from this method regarding *B. ranarum* (**Figure 4.11**) showed that all tested *Allium* species showed an antifungal activity against this pathogen. Among the tested *Allium* samples, *A. stipitatum* with the average MIC of 0.2mg/mL (SD=0.2) showed the highest antifungal activity, followed by *A. sativum* with the average MIC of 0.3mg/mL (SD=0.1). A rather high efficacy was also seen in the results gained by *A. pictistamineum* and *A. paniculatum* subsp. *paniculatum* showing the average minimum inhibitory concentration of 1.2mg/mL (SD=0.2) and 1.9mg/mL (SD=1.0) respectively. *A. moly* also

presented a significant effectiveness toward *B. ranarum* with the mean MIC of 2.2mg/mL (SD=1.0). The MIC calculated for miconazole, 0.04mg/mL (SD=0.2), was higher than all *Allium* samples tested against *B. ranarum*.

Results obtained from the antifungal activity of the *Allium* species against *C. neoformans* (**Figure 4.12**) were also positive for all the tested samples. The highest MIC, <0.08mg/mL (breakpoint out of the measured range, i.e. 10-0.08mg/mL), belonged to *A. stipitatum* followed by *A. sativum* and *A. pictistamineum* presenting the MIC 0.2mg/mL (SD=0.0) and 1.5mg/mL (SD=0.4) subsequently. The MIC calculated for miconazole, 0.05mg/mL (SD=0.2), was higher than most of the *Allium* samples tested against *C. neoformans*, and almost comparable to the MIC gained by *A. stipitatum*.

The next pathogen, *E. floccosum* (**Figure 4.13**), showed the highest susceptibility towards *A. stipitatum* and *A. sativum*, with the minimum inhibitory concentration of <0.08mg/mL. In this case, since the breakpoint was less than the microdilution endpoint (10-0.08mg/mL), the MIC could not be clearly determined. Other effective species are *A. pictistamineum*, *A. moly,* and *A. paniculatum* subsp. *paniculatum* with an average MIC of 0.1mg/mL (SD=0.0), 0.2mg/ml (SD=0.1) and 0.3mg/mL (SD=0.1) respectively. In general, all *Allium* species had a significant effect on *E. floccosum* having a MIC less than 1.0mg/mL (**Table 6**).

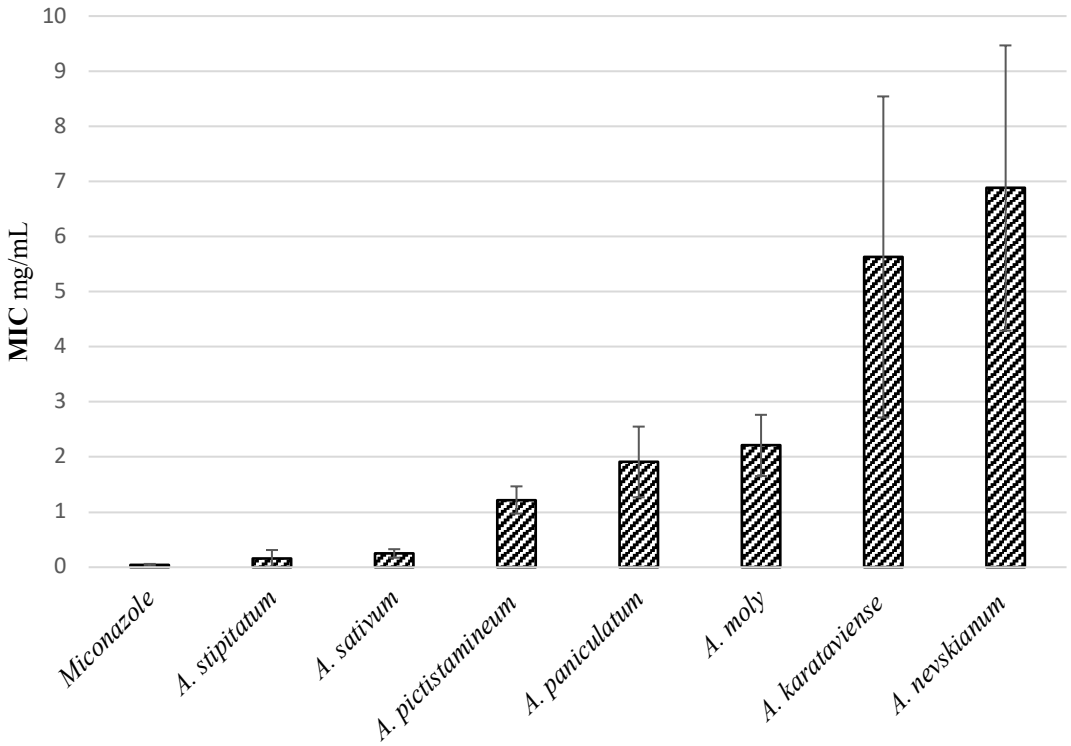


Figure 4.11 A diagram comparing MIC (mg/mL) of miconazole and the *Allium* species against *B. ranarum*

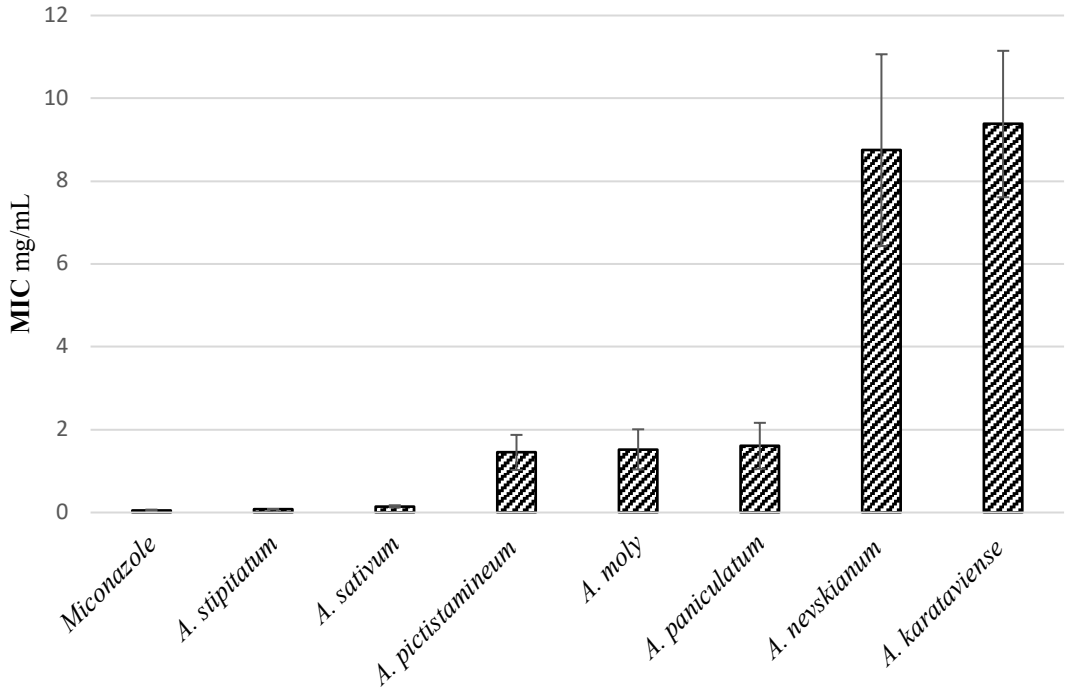


Figure 4.12 A diagram comparing MIC (mg/mL) of miconazole and the *Allium* species against *C. neoformans*

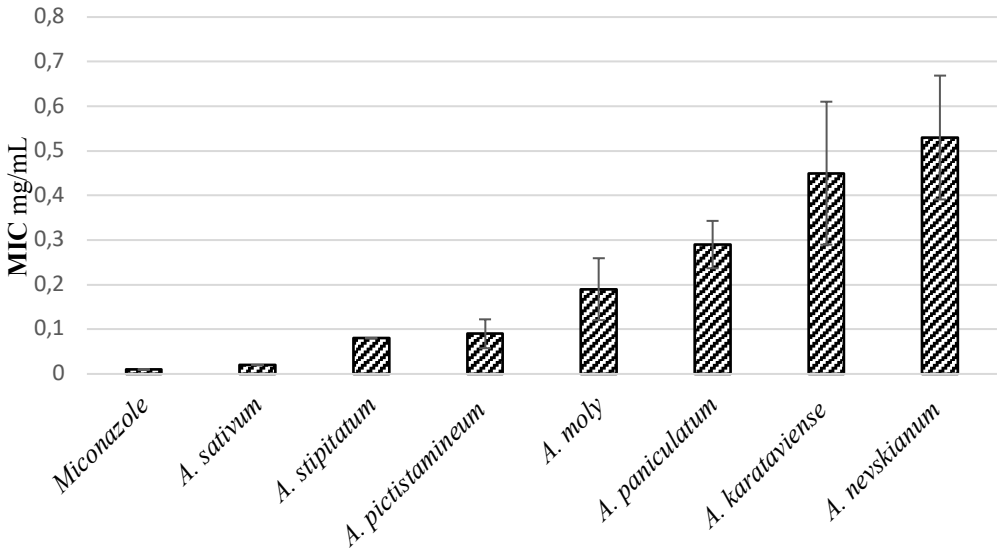


Figure 4.13 A diagram comparing MIC (mg/mL) of miconazole and the *Allium* species against *E. floccosum*

Name	Subgenus	MIC (mg/mL)	Standard Deviation (mg/mL)
Basidiobolus ranarum			
Miconazole		0.04	0.0
A. stipitatum	*Melanocrommyum*	0.2	0.2
A. sativum	*Allium*	0.3	0.1
A. pictistamineum	*Allium*	1.2	0.2
A. moly	*Amerallium*	2.2	0.6
A. paniculatum subsp. *paniculatum*	*Allium*	1.2	0.6
A. nevskianum	*Melanocrommyum*	6.9	2.6
A. karataviense	*Melanocrommyum*	5.6	2.9
Cryptococcus neoformans			
Miconazole		0.1	0.0
A. stipitatum	*Melanocrommyum*	0.1	0.0
A. sativum	*Allium*	0.2	0.0
A. pictistamineum	*Allium*	1.5	0.4

Name	Subgenus	MIC (mg/mL)	Standard Deviation (mg/mL)
A. moly	*Amerallium*	1.5	0.5
A. paniculatum subsp. *paniculatum*	*Allium*	1.6	0.6
A. nevskianum	*Melanocrommyum*	8.8	2.3
A. karataviense	*Melanocrommyum*	9.4	1.8
Epidermophyton floccosum			
Miconazole		0.01	0.0
A. stipitatum	*Melanocrommyum*	0.1	0.0
A. sativum	*Allium*	0.1	0.0
A. pictistamineum	*Allium*	0.1	0.0
A. moly	*Amerallium*	0.2	0.1
A. paniculatum subsp. *paniculatum*	*Allium*	0.3	0.1
A. nevskianum	*Melanocrommyum*	0.5	0.1
A. karataviense	*Melanocrommyum*	0.5	0.2

Table 6 Detailed results of the minimum inhibitory concentrations and their standard deviations regarding *B. ranarum, C. neoformans* and *E. floccosum*

4.3.2 Disk diffusion test

As seen in diagram 2, *B. ranarum* (**Figure 4.14**) was mostly susceptible to *A. stipitatum* with the average inhibition zone of 28mm (SD= 2) in diameter. *A. pictistamineum* and *A. sativum* caused smaller inhibition zones than *A. stipitatum* with the average ZOI of 25mm (SD=1) and 21mm (SD= 3) respectively. On the other hand, *A. nevskianum* and *A. karataviense* were unable to generate any inhibition zone regarding *B. ranarum.* In comparison, *C. neoformans* (**Figure 4.15**) was mostly resistant toward *Allium* species with 6 samples resulting in an inhibition zone. This pathogen shows highest susceptibility towards *A. stipitatum* with a ZOI of 58mm (SD= 2), followed by *A. pictistamineum, A. moly* and *A. sativum* with average zones of inhibition of 30mm (SD=2), 29mm (SD=2) and 28mm (SD=2) respectively. On the other hand, *E. floccosum* (**Figure 4.16**) demonstrated significant sensitivity toward *A. pictistamineum* with an average zone of inhibition of 71mm (SD= 3) in diameter, following by *A. stipitatum* and *A. sativum* with the average ZOI of 67mm (SD=2) and 60mm (SD=3) subsequently. This pathogenic fungus

was the most susceptible to all tested pathogens towards *Allium* samples. More detailed results regarding the ZOI is presented in **Table 7.**

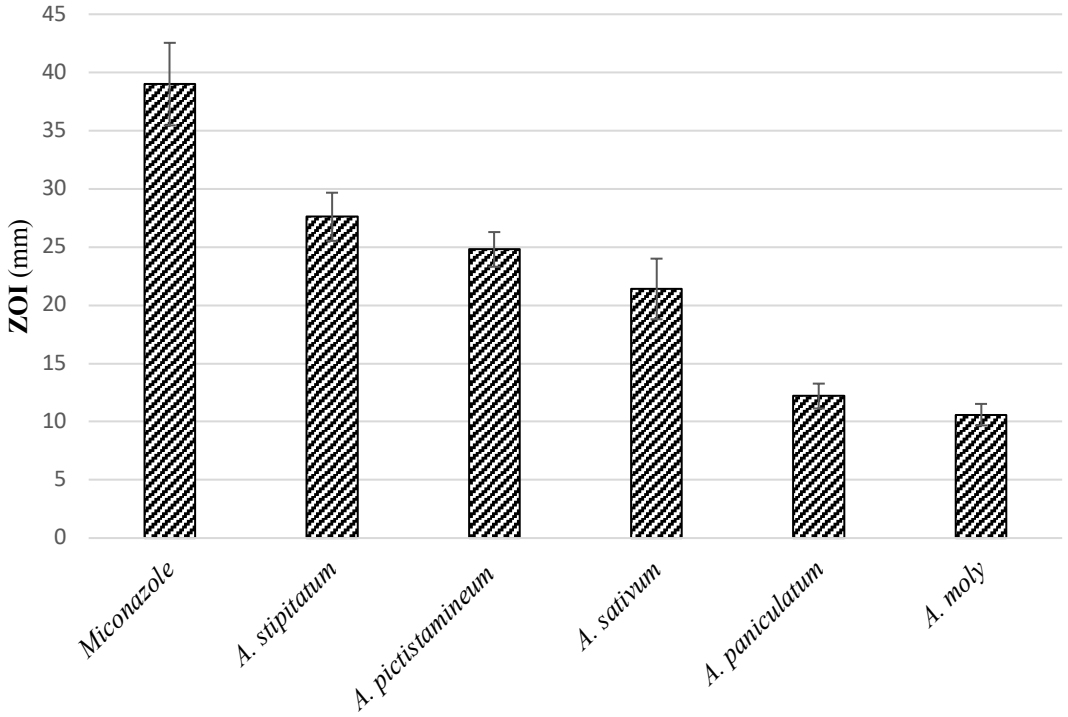


Figure 4.14 A diagram comparing ZOI (mm) of miconazole and the *Allium* species against *B. ranarum*

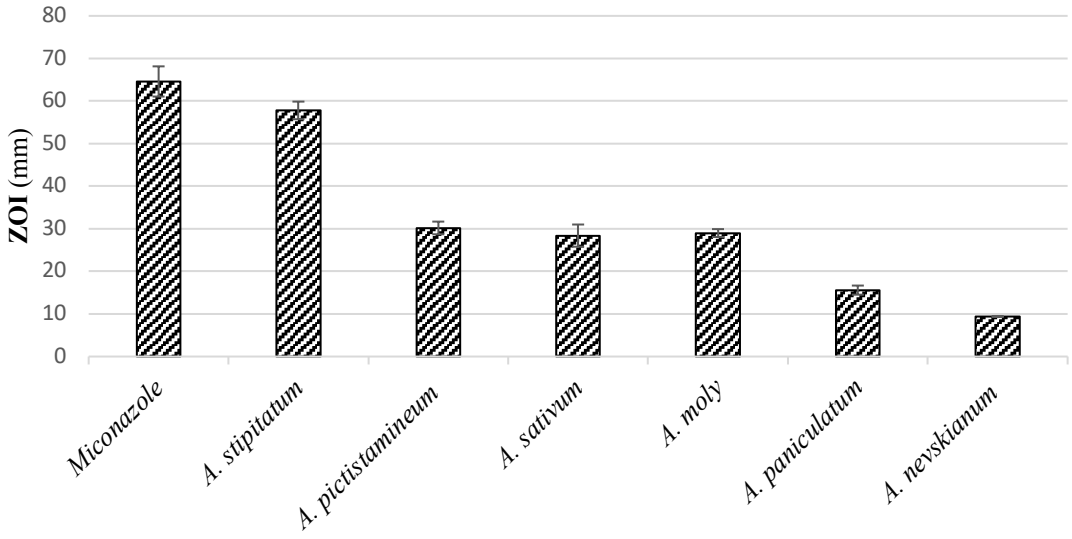


Figure 4.15 A diagram comparing ZOI (mm) of miconazole and the *Allium* species against *C. neoformans*

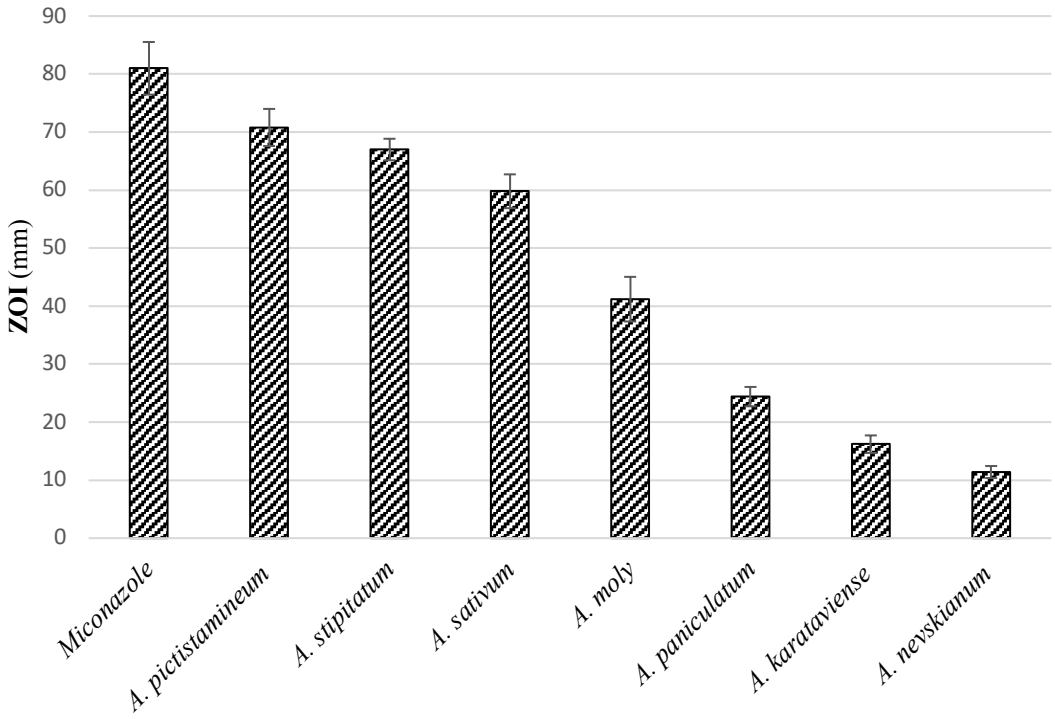


Figure 4.16 A diagram comparing ZOI (mm) of miconazole and the *Allium* species against *E. floccosum*

Name	Subgenus	ZOI (mm)	Standard Deviation (mm)
Basidiobolus ranarum			
Miconazole		39	4
A. stipitatum	*Melanocrommyum*	28	2
A. sativum	*Allium*	21	3
A. pictistamineum	*Allium*	25	1
A. moly	*Amerallium*	11	1
A. paniculatum subsp. *paniculatum*	*Allium*	12	1
Cryptococcus neoformans			
Miconazole		65	3
A. stipitatum	*Melanocrommyum*	58	2
A. sativum	*Allium*	28	2
A. pictistamineum	*Allium*	30	2
A. moly	*Amerallium*	29	2

Name	Subgenus	ZOI (mm)	Standard Deviation (mm)
A. paniculatum	*Allium*	16	1
A. nevskianum subsp. *paniculatum*	*Melanocrommyum*	9	1
Epidermophyton floccosum			
Miconazole		81	5
A. stipitatum	*Melanocrommyum*	67	2
A. sativum	*Allium*	60	3
A. pictistamineum	*Allium*	71	3
A. moly	*Amerallium*	41	4
A. paniculatum subsp. *paniculatum*	*Allium*	24	2
A. nevskianum	*Melanocrommyum*	11	1
A. karataviense	*Melanocrommyum*	16	2

Table 7 Detailed results of zones of inhibition and their standard deviations regarding *B. ranarum, C. neoformans* and *E. floccosum*

4.3.3 Double-dish chamber

Results gained from this method demonstrated that all the examined *Allium* samples showed an antifungal activity regarding their volatile compounds against at least one of the tested fungi. The largest zone of inhibition was made by *A. sativum* and *A. pictistamineum against E. floccosum* were absolutely no fungal growth was detected. *A. sativum* by causing the largest ZOIs in comparison to all the other *Allium* species proved to have the most effective VSCs (**Figure 4.17**), following by *A. stipitatum* and *A. pictistamineum*. On the other hand, as expected as due to the results gained via other methods, *A. nevskianum* and *A. karataviense* appeared weaker than other *Allium* species by only affecting the growth of *E. floccosum.* More detailed results regarding the ZOI is presented below (**Table 8**).

Name	*B. ranarum*	*C. neoformans*	*E. floccosum*
A. sativum	45-51 mm	60-65 mm	no growth
A. nevskianum	-	-	25-28 mm
A. pictistamineum	15-18 mm	25-28 mm	no growth
A. karataviense	-	-	44-47 mm
A. stipitatum	-	44-48 mm	42-45 mm
A. moly	24-27 mm	29-34 mm	75-80 mm
A. paniculatum subsp. *paniculatum*	16-18 mm	31-33 mm	55-57 mm

Table 8 Zone of inhibition (ZOI) diameter in mm of the *Allium* species antifungal activity regarding the volatile compounds against the human pathogenic fungi

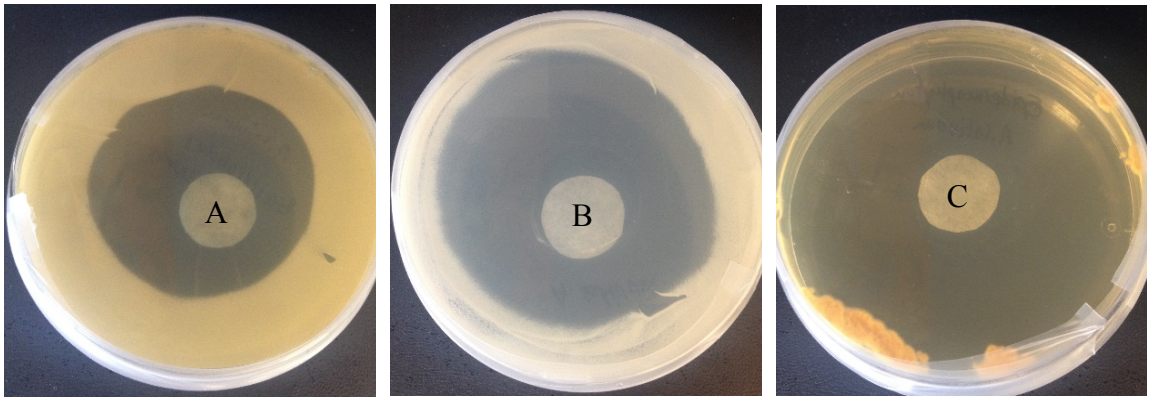


Figure 4.17 Double-dish chamber results of *A. sativum* (A) on *B. ranarum*, (B) on *C. neoformans* and (C) on *E. floccosum* after 72hr of incubation

4.3.4 Summarized results regarding human pathogenic fungi

The antifungal effects of 7 *Allium* species crude extracts were tested against *Basidiobolus ranarum*, *Cryptococcus neoformans* and *Epidermophyton floccosum*. In order to achieve this, agar microdilution method, disk diffusion test and double-dish chamber were used. Comparing the average effect of all the sample exhibits a meaningful relation between all used methods (**Table 9**). *A. stipitatum* with an average MIC of 0.1mg/ml against three human pathogenic fungi presented the highest average minimum inhibitory concentration among all the tested *Allium* samples, while showing the largest

zone of inhibition regarding the disk diffusion test with the average ZOI of 22.4mm as well. On the other hand, *A. nevskianum*, with an average minimum inhibitory concentration of 5.38mg/ml exhibited the highest MIC among all the tested samples, also having the smallest average radius concerning disk diffusion method. The average results gained by the double-dish chamber differed from other calculated outcomes, in a way that *A. sativum* showed the largest ZOI with 67.0mm in diameter, which is quote explainable, showing that the more antifungal activity of this *Allium* species is caused by the volatile sulfur compounds. More detailed results can be seen in **Table 9**.

	Microdilution MIC [mg/ml]	**Disk diffusion radius [mm]**	**Double-dish chamber diameter [mm]**
A. stipitatum	0.1	22	30
A. sativum	0.1	14	67
A. pictistamineum	0.9	18	45
A. paniculatum subsp. *paniculatum*	1.3	6	35
A. moly	1.3	11	45
A. karataviense	5.3	2	15
A. nevskianum	5.4	2	9

Table 9 Summarized results regarding human pathogenic fungi. An average of the results gained by the effect of each *Allium* species against all three human pathogenic fungi samples is calculated for each method.

4.4 *Allium stipitatum*, Identification of active substances

Based on the screening of 51 crude extracts of plants from genus *Allium*, *A. stipitatum*, the famous Persian “Mu-sir” which displayed various levels of anti-fungal activities, was selected to assess the further investigations.

4.5 Bioassay-guided fractionation and isolation of the active compounds (Prep HPLC).

Bioassay-guided fractionation is a common approach for studying crude plant extract. This technique is based on the screening of fractions that show biological activity towards microorganisms. The above technique has been applied to isolate and purify the bioactive compounds in *Allium stipitatum* and the structure elucidation of the compounds responsible for the anti-fungal activity. Therefore, *A. stipitatum* ethyl acetate crude extract was dissolved in methanol and subjected to preparative HPLC. From the preparative chromatographic separation, 5 prominent fractions were collected, dried and labeled as fractions 1-5 as shown in the preparative HPLC chromatogram in **Figure 4.18**. All 5 fractions were subjected to bioactivity testing with the concentration of 10mg/mL using drop test screening method. Significant inhibitions were observed with the micro-fractionated *A. stipitatum* samples. 2 out of 5 fractions were found to possess bioactivity. Fraction 2 acquired during the retention time 19.95min was toxic to all the test microorganisms while fraction 3 eluting at a retention time of 25.37min proved to be the most potent. More detailed measurements concerning diameter range of ZOI regarding the drop test screening method of fraction 2 and 3 can be seen in **Table 10**. Both effective fractions were then analyzed using HPLC and were found to be quite pure compounds. The preparative HPLC chromatogram of the *A. stipitatum* EtAc extract at wavelength 254 nm is as shown in **Figure 4.18.**

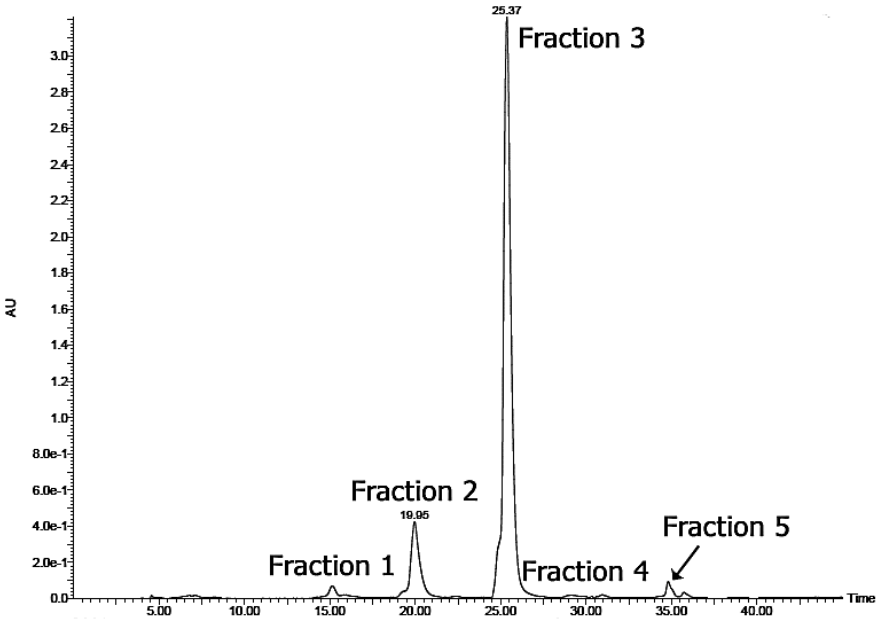


Figure 4.18 Preparative HPLC-UV chromatogram (λ= 254nm) of *Allium stipitatum* ethyl acetate extract.

	Fraction 2	**Fraction 3**
Mucor hiemalis	-	28-33
Aspergillus flavus	20-26	44-55
Aspergillus niger	20-30	47-52
Penicillium italicum	16-28	48-60
Basidiobolus ranarum	23-29	50-56
Cryptococcus neoformans	35-42	54-61
Epidermophyton floccosum	40-44	59-66

Table 10 Diameter range of ZOI regarding the drop test screening method of fraction 2 and 3. The concentration of the fractions were set to 10mg/ml. Zone of inhibition ranges were measured in mm.

4.6 LC-MS adducts general Interpretation of *A. stipitatum*

In order to get a complete overview of all the compounds masses analysis found in *A. stipitatum*, a liquid chromatography-mass spectrometry test was performed on its crude extract. The method by which the LC-MS test was conducted can be seen in **Table 11**. As seen in **Table 12**, 25 different compounds and their related adduct ions was found in *A. stipitatum* crude extract. Compound 10 with the actual mass of 173 (RT= 11.23) (**Figure Figure 4.19**) and compound 14 with the actual mass of 219 (13,81 / 14,47) (**Figure 4.20**) proved to be the most active ones regarding the bioassay-guided fractionation of the compounds. But there are still other compounds remained for further investigations regarding other possible bioactivities.

Time [min]	**MeOH [%]**	**H_2O [%]**
Initial	25	75
25	100	0

Table 11 The method used for liquid chromatography-mass spectrometry test

	RT	Max. cps.	M/Z	M+H	M+NH_4	M+Na	+74	M+K	Others
1	2,28	2,3e5	362	363	-	385	-	-	407 (M+2Na-H)
2	3-4	2,8e5	194	195	-	217	268	-	-
3	3-5	4,6e5	238	239	256	261	312	-	-
4	5,30	7,5e5	282	283	300	305	356	321	-
5	6,50	8,5e5	326	327	344	349	400	365	-
6	7,62	1,3e6	370	371	388	393	444	409	-
7	8,45	1,1e6	414	415	432	437	488	453	-
8	8,69 / 9,65	6,3e5	458	459	476	481	-	497	-
9	10,85	1,8e5	546	547	564	569	620	585	-
10	**11,23**	**3,0e6**	**173**	**174**	**-**	**196**	**247**	**-**	**-**
11	11,25	1,0e6	109	110	127	-	-	-	-
12	12,57 / 13,66	4,9e5	313	314	-	336	-	-	-
13	13,83 / 14,39	1,6e6	91	92	109	-	-	-	-
14	**13,81 / 14,47**	**2,4e6**	**219**	**220**	**-**	**242**	**293**	**258**	**439 (2M+H) / 461 (2M+Na)**
15	15,86	1,8e5	344	345	362	367	418	383	-
16	16,31	1,2e6	209	210	228	-	-	-	-
17	16,75	1,2e5	462	463	480	485	536	-	-
18	18,48	2,1e5	123	124	-	-	-	-	156 (M+CH_3OH+H) / 206 (M+2ACN+H)
19	19,19	1,4e5	312	313	330*	335	386	-	-
20	19,46	1,7e5	134	135	-	-	-	-	179 (M+2Na-H)
21	21,63	1,3e6	211	212	-	-	287	-	-
22	21,06	7,6e5	256	257	-	279*	-	296	-
23	23,94	5,3e5	323	324	341	346	-	-	-
24	23,81	2,4e5	253	254		276	-	-	-
25	24,86	7,0e5	332	333	355*		-	-	379 (M+ACN+H)

Table 12 LC-MS adduct ions results of *A. stipitatum* crude extract

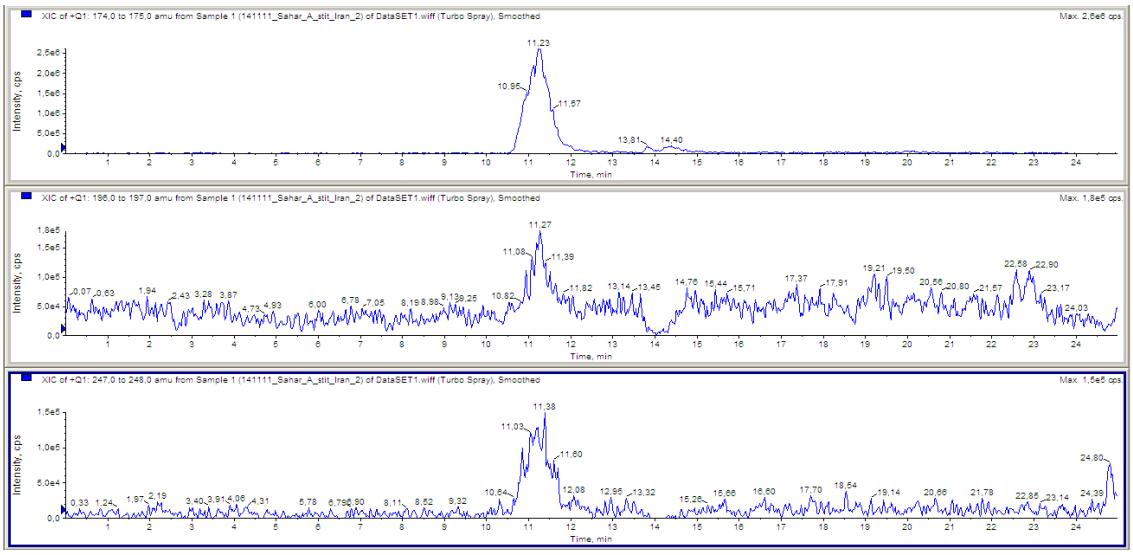


Figure 4.19 Extracted-ion chromatogram (XIC) of M/Z: 174 adducts

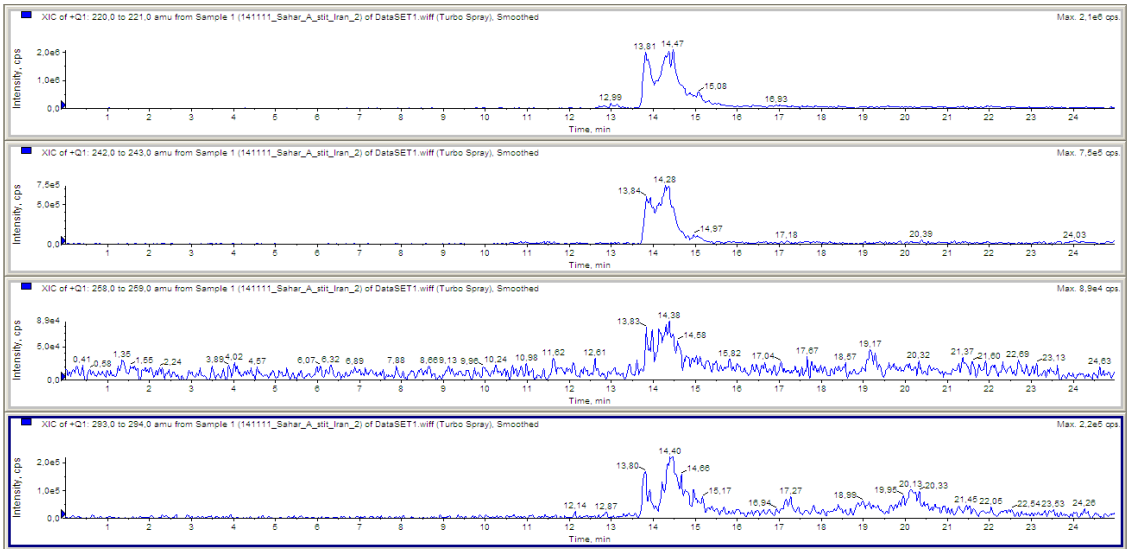


Figure 4.20 Extracted-ion chromatogram (XIC) of M/Z: 220 adducts

4.7 Chemical nature of the bioactive fractions

The bioactive compounds were then identified and determined on the basis of LC-ESI/MS, HR-MS, and 1H NMR data. Both effective fractions were subjected to LC-ESI/MS. The mass spectrum from those acquired during the retention time of 19.60min and 19.01min showed prominent $[M+H]^+$ ion peaks of m/z 174 and m/z 220 respectively. HR-MS suggested the molecular formula of $C_6H_8NOS_2$ and $C_7H_{10}NOS_3$ for fraction 2 and 3 subsequently. The spectrum of 1H NMR regarding fraction 2 indicated an ABCD aromatic system based on 4 hydrogens (δ 8.34, 7.86, 7.47 and 7.31), and a singlet peak at δ 2.47

correlating to a deshielded methyl group due to the inductive effect from the sulfur atoms. On the other hand, the spectrum belonging to fraction 3 was quite comparable to the NMR results belonging to fraction 2, namely showing the same hydrogen aromatic ring and the deshielded methyl group. The presence of a methylene singlet (δ 3.98) was the only noticeable difference. The isolated bioactive compounds were compared with the reported literature and were confirmed to be the previously described 2-(Methyldithio)pyridine-N-oxide and 2-[(Methylthiomethyl)dithio]pyridine-N-oxide, which were first found in *A. stipitatum* [146]. The chemical structures of fractions 2 and 3 are as seen in **Figure 4.21** and **Figure 4.22**, respectively.

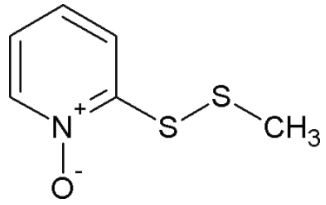


Figure 4.21 Chemical structure of 2-(Methyldithio)pyridine-*N*-oxide (fraction 2)

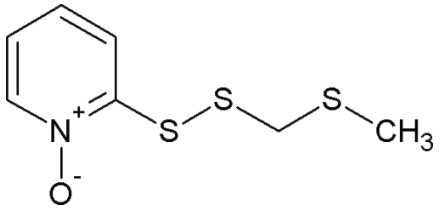


Figure 4.22 Chemical structure of 2-[(Methylthiomethyl)dithio]pyridine-*N*-oxide (fraction 3)

5. DISCUSSION

Human being has always believed in the mysterious healing power of nature. Today we are inventing medicine in the laboratory, and in order to do that, we need thousands of years of trial and error. Plant and nature are the foundation of modern medicine. It is sometimes believed that these ancient folk remedies from thousands of years ago has nothing left to teach us, but now that the synthetic drugs have reached a plateau, scientists have begun to realize that maybe in order to go forward, we need to go backward and discover what nature has to offer us.

We are surrounded by many plants that could serve the future of medicine. Lots of these bizarre folk medicines are in fact pharmaceutically potentially valuable. And improving our understanding of how these ancient remedies work is valuable to many areas of medicine.

Since the early 1970s, agriculture has struggled with evolving pathogen resistance towards antimicrobial disease control agents. Increased requirement for repeated chemical applications, development of pesticide cross-resistance, and disease resistance management strategies have overshadowed the use of agricultural chemicals [147]. Widespread use of pesticides has significant drawbacks including cost, pesticide residues, and threats to human health and environment [148]. Scientists are currently attempting to control

agricultural pests with effective alternative controls. In addition, the desire for safer pesticides with lower environmental toxicity is a major public concern [149].

Although the members of *Allium* family differ in taste, form, and color, they seem be rather close in biochemical, phytochemical and nutraceutical content [150]. However, these investigations demonstrate that there are significant differences between the tested species. As stated in the introduction section, sulfur compounds are believed to be the active principle of *Allium*. In the intact bulb, cysteine sulfoxides are the dominating compounds. It could be demonstrated in previous research that the pattern of cysteine sulfoxides **Figure 1.4** and consequently also the pattern of volatile sulfur compounds differ significantly between species [55, 151-154]. The cysteine sulfoxide methiin occurs in nearly all investigated *Allium* species were as alliin is typically occurred in the subgenera *Allium*, *Butomissa*, *Polyprason* (punctually) and *Anguinum* [153]. On the other hand, isoalliin is most characteristic for the subgenus *Cepa*, but also *A. ampeloprasum* belonging to the subgenus *Allium* shows rather high amounts of isoalliin. The here investigated *Allium* species mainly contain 'usual' cysteine sulfoxides with aliphatic side chains. Members of the subgenus *Melanocrommyum* also show a high amount of methiin as well as the sulfur-containing pyridine N-oxides which are the characteristics for *A. stipitatum* [155].

If the alliinase acts the cysteine sulfoxides, thiosulfinates with the general formula $Rest_1$-S-SO-$Rest_2$ were formed. For the subgenus *Cepa*, also the so-called 'Lachrymatory factor' (LF) can be the major volatile sulfur product out of isoalliin. For *A. sativum*, the major thiosulfinate is allicin with the formula Allyl-S-SO-Allyl. As shown in the previous investigations, *Allium* species usually contain a mixture of different cysteine sulfoxides, which means that after alliinase reaction the two rests of the thiosulfinate are unequal. The number of possible thiosulfinates follows the rule $x = n^2$ (in case of not taking S-stereo-chemistry into consideration), where x is the number of thiosulfinates and n the number of cysteine sulfoxides. This means that the pattern of thiosulfinates can be deduced from the pattern of cysteine sulfoxides, which have been previously investigated for numerous wild species [55, 153]. Because of the possible LF-formation, the situation is getting even more complex, if isoalliin is present.

Thiosulfinates are believed to be the most effective antimicrobial principle of *Allium*. However, it must be considered that these compounds are instable and will result mostly in different polysulfides with the general formula $Rest_1$-$(S)_n$-$Rest_2$ [55, 61]. In case that the

rest are allyl (deduced from alliin), there is still a significant bioactivity [54]. Sulfides deduced from isoalliin seems to be less antimicrobial active, whereas pyridyl-containing compounds derived from various pyridine N-oxides in *Allium stipitatum* have proved to have a high bioactivity [146]. However, it must be pointed out that also saponins, as well as flavonoids, can be extracted partially by EtOAc, meaning that besides the sulfur compounds also other bioactives can cause antifungal activity.

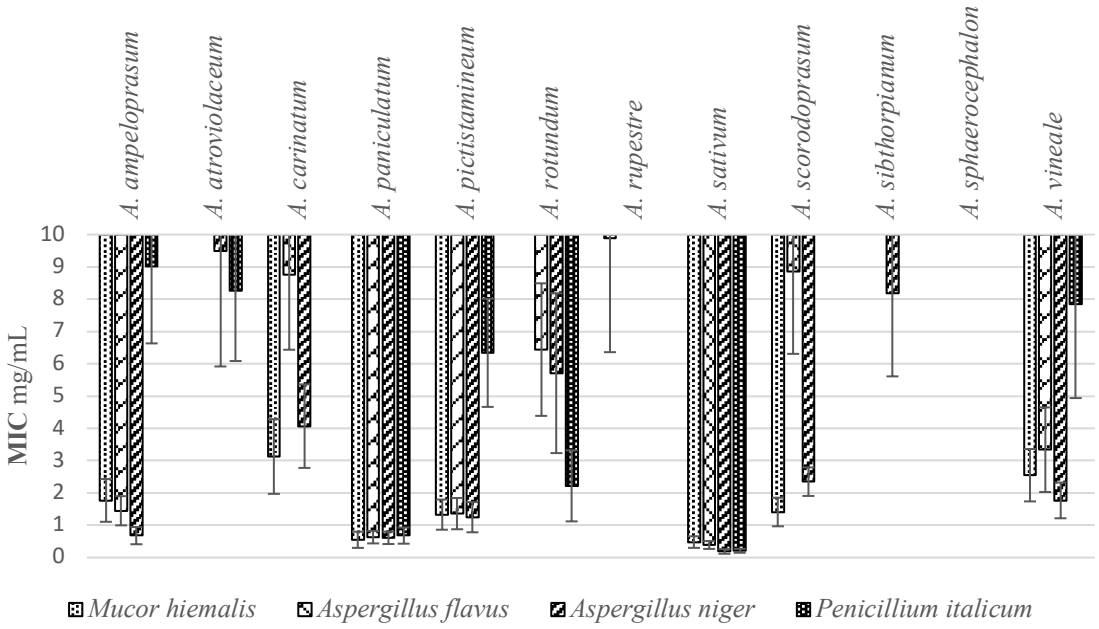


Figure 5.1 A diagram comparing MIC (mg/ml) of the *Allium* species belonging to the subgenus *Allium* against all the plant pathogenic fungi

Over 300 species of the genus *Allium* belong to subgenus *Allium* with its typical representative garlic. Regarding the relative amounts of cysteine sulfoxides in bulbs of the members of this subgenus, studies [155] have shown that almost all members of the subgenus *Allium* contain methiin, alliin, and isoalliin while the pattern of cysteine sulfoxides varies significantly among the members of this subgenus. It can also be mentioned that the spicy taste and health benefits of *Allium* species is related to the occurrence of alliin and isoalliin.

As seen in **Figure 5.1,** almost all the members of the subgenus *Allium* show an antifungal activity toward at least one pathogenic species. *A. sativum* can be named as the most active species of this subgenus which has proved to contain a relatively high amount of alliin.

Surprisingly, *A. paniculatum* subsp. *paniculatum* and *A. pictistamineum* also presented a high antifungal activity toward all the tested plant pathogens. As summarized in **Figure 5.1** the MIC calculated for these two *Allium* species is quite comparable with the minimum inhibitory concentration showed by *A. sativum*. This result shows the importance of further investigations on *A. paniculatum* subsp. *paniculatum* and *A. pictistamineum.*

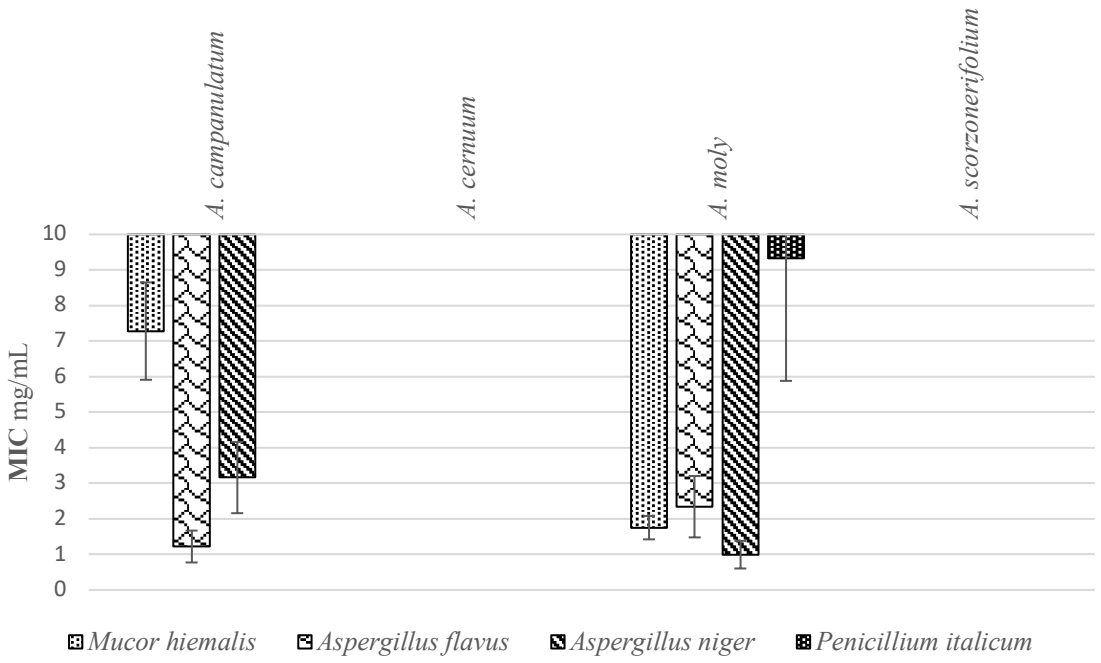


Figure 5.2 A diagram comparing MIC (mg/ml) of the *Allium* species belonging to the subgenus *Amerallium* against all the plant pathogenic fungi

Out of 4 *Allium* species belonging to the subgenus *Amerallium*, two of them demonstrated a MIC more than 10mg/mL towards at least three plant pathogenic fungi (**Figure 5.2**). Generally, members of the subgenus *Amerallium* contain methiin, isoalliin and propiin [155], but it should always be taken into consideration that different ontogenetic stages of collected plants can lead to different amounts of cysteine sulfoxide, and therefore different antimicrobial activity.

Figure 5.3 A diagram comparing MIC (mg/ml) of the *Allium* species belonging to the subgenus *Anguinum* against all the plant pathogenic fungi

Only one member of the subgenus *Anguinum* was tested against the pathogenic fungi (**Figure 5.3**). *A. victorialis* did not show any minimum inhibitory concentration less than 10mg/mL. Unfortunately, not enough data regarding the amount of cysteine sulfoxides of *A. victorialis* or generally the subgenus *Anguinum* were available in the previous studies.

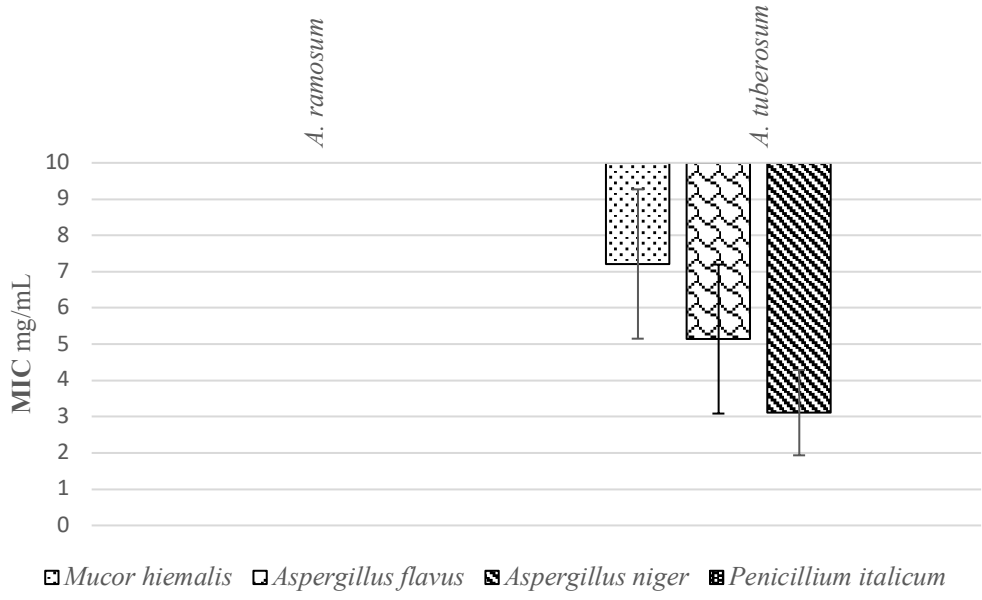


Figure 5.4 A diagram comparing MIC (mg/ml) of the *Allium* species belonging to the subgenus *Butomissa* against all the plant pathogenic fungi

Two members of the subgenus *Butomissa* (**Figure 5.4**) were available to be tested against the pathogenic fungi, among which, one of them demonstrated an MIC below 10mg/mL

toward 3 out of 4 tested pathogens. According to the analysis of the total amount of cysteine sulfoxides [155], a significant total amount of flavor precursors were detected in *A. tuberosum,* among which methiin was found to be the main cysteine sulfoxide in the bulbs of this *Allium* species.

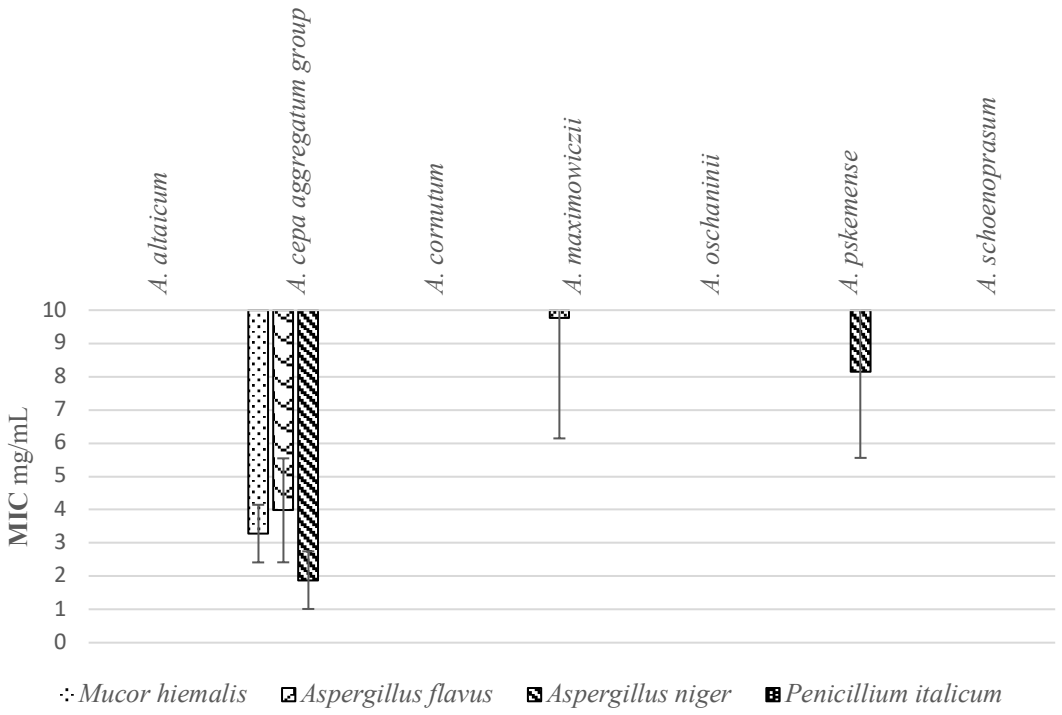


Figure 5.5 A diagram comparing MIC (mg/ml) of the *Allium* species belonging to the subgenus *Cepa* against all the plant pathogenic fungi

Subgenus *Cepa* contains approximately 30 rhizomatous species, out of which 7 samples (**Figure 5.5**) were tested. 3 species, i.e., *A. cepa aggregatum* group, *A. maximowiczii,* and *A. pskemense* showed an antifungal activity less than 10mg/mL towards at least one of the pathogenic fungi. Generally, members of the subgenus *Cepa* are rich in GPC (Glutamyl-Propenyl-Cysteinsulfoxid), isoalliin and methiin. It is interesting to know that alliin has also been reported from all the wild *Allium* samples with the exception of *A. oschaninii*. It must also be mentioned that the amount of alliin available in the species belonging to subgenus *Cepa* is relatively low comparing to the isoalliin. Methiin has also been proved to have a relative low concentration. On the other hand, propiin has also been found in some species such as *A. oschaninii* [156].

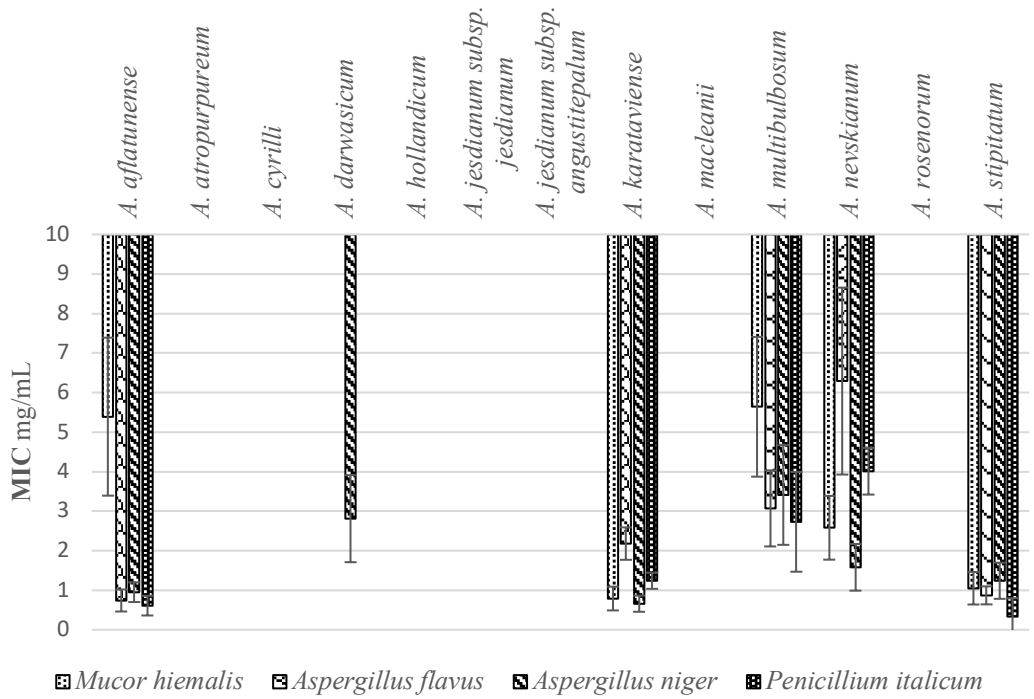


Figure 5.6 A diagram comparing MIC (mg/ml) of the *Allium* species belonging to the subgenus *Melanocrommyum* against all the plant pathogenic fungi

Melanocrommyum is a very large subgenus containing around 140 species divided into 15 sections. Analysis has shown that species belonging to this subgenus contain only a small amount of cysteine sulfoxides. Therefore the typical smell of garlic or onion is absent in them [153]. Like subgenus *Cepa* and *Polyprason*, members of the subgenus *Melanocrommyum* are rich in methiin. On the other hand, some *Melanocrommyum* species show a characteristic red coloration when damaged. Depending on the species, this coloration occurred in seconds or minutes and became more intense. It was found that this red coloration was caused by a cysteine sulfoxide called dithiopyrrole (**Figure 5.7**), and it is assumed that an alliinase-like enzyme is also involved in the formation of this compound [155].

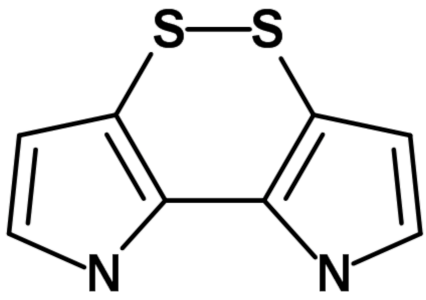


Figure 5.7 Isolated dithiopyrrole ((+)-S- (3-pyrrolyl)-L-cysteine sulfoxide) from Melanocrommyum species

However, dithiopyrrole does not appear to be formed in all species of the subgenera Melanocrommyum, as not all species show this red coloration. In the current study, out of 13 tested *Allium* samples, 5 contained dithiopyrrole, i.e., *A. hollandicum*, *A. jesdianum* subs. *jesdianum*, *A. jesdianum* subsp. *angustitepalum*, *A. macleanii,* and *A. rosenorum*. Interestingly, none of these species showed any antifungal activity towards the tested pathogenic fungi. Other tested members of the subgenus Melanocrommyum except for *A. atropurpureum* and *A. cyrilli* demonstrated a rather high antifungal activity towards the tested pathogenic species. It should also be mentioned that the inhibitory effect of *A. stipitatum* and *A. karataviense* were significantly high compared to other *Allium* species. *A. stipitatum* contains different bioactive organosulfur compounds such as dipyrithione and other related sulfur-containing pyridine N-oxides, the bioactivity effects of which, have already been proved [146]. *A. karataviense* is also rich in propiin. More detailed results can be seen in **Figure 5.6**.

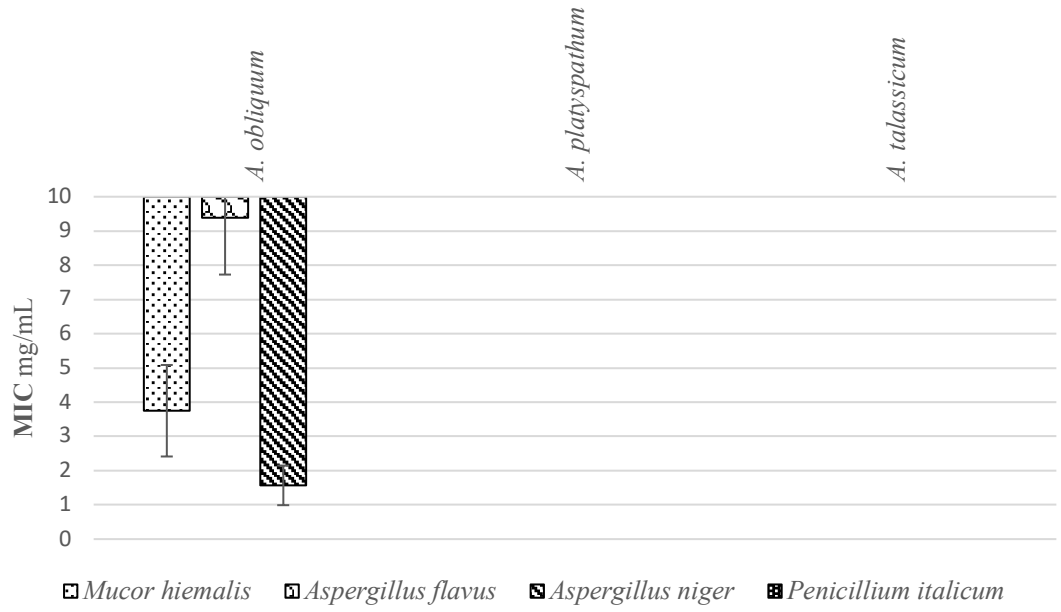


Figure 5.8 A diagram comparing MIC (mg/ml) of the *Allium* species belonging to the subgenus *Polyprason* against all the plant pathogenic fungi

Subgenus *Polyprason* contains approximately 50 species divided into 4 sections. In this study, 3 species belonging to this subgenus were tested regarding their antifungal activity. As already mentioned above, members of the subgenus *Polyprason* are rich in methiin as well as *Cepa* and *Melanocrommyum* species, while alliin isoalliin were found in small concentrations. As seen in **Figure 5.8**, only *A. obliquum* showed an antifungal activity less than 10mg/mL towards 3 out of 4 phytopathogenic fungi.

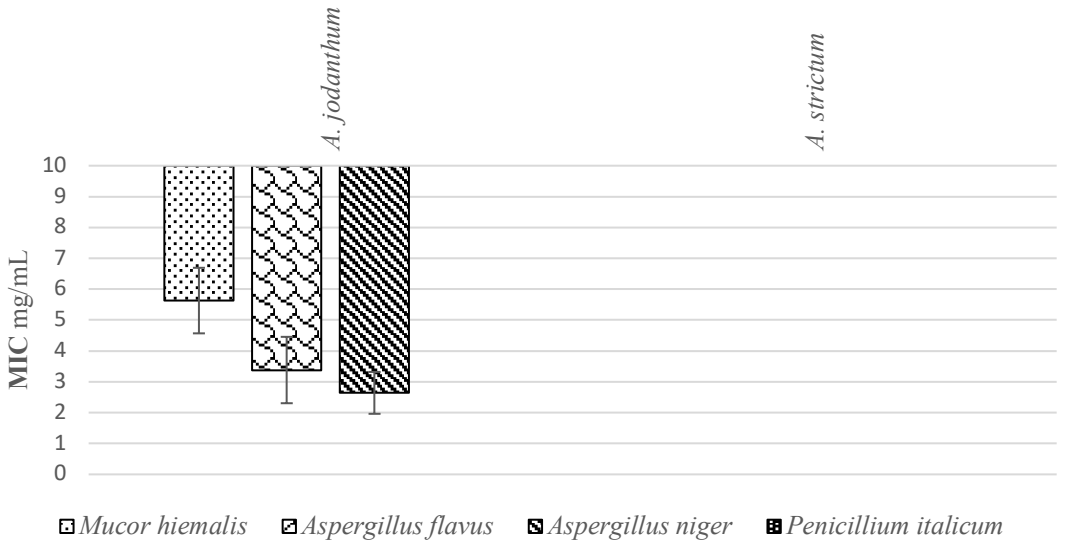


Figure 5.9 A diagram comparing MIC (mg/ml) of the *Allium* species belonging to the subgenus *Reticulatobulbosa* against all the plant pathogenic fungi

Around 80 *Allium* species are classified in subgenus *Reticulatobulbosa,* among which, 2 samples were used in this study. Previous analysis has shown that more than 50% of the total amount of cysteine sulfoxides in the members of the subgenus *Reticulatobulbosa* is methiin, and alliin, as well as isoalliin, can be found only in a very small amount [156]. As seen in **Figure 5.9**, *A. jodanthum* demonstrated an MIC less than 10mg/mL towards all the tested fungal species except for *Penicillium italicum*.

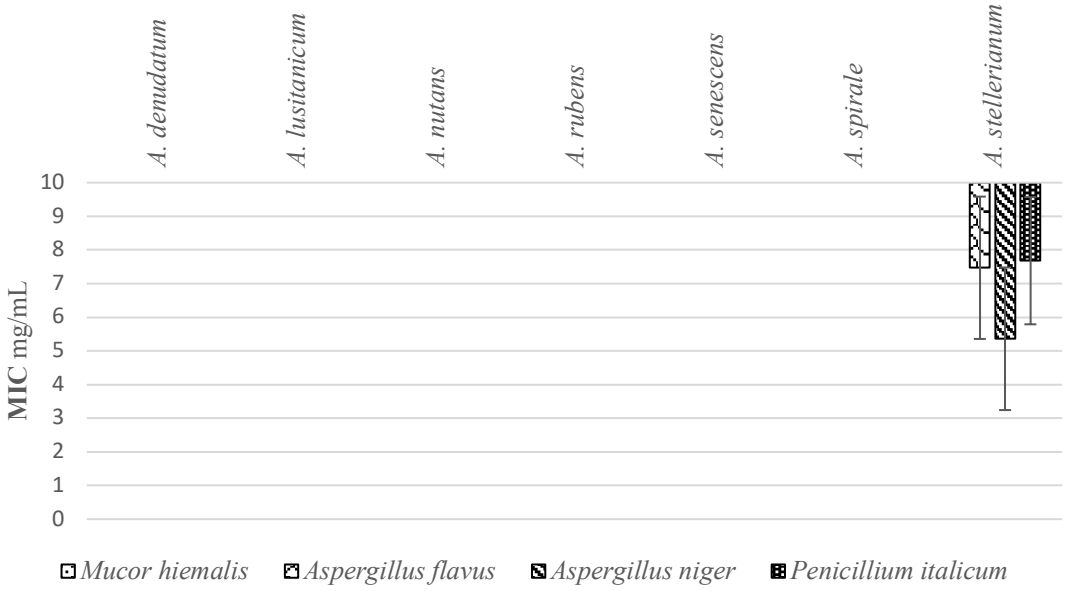


Figure 5.10 A diagram comparing MIC (mg/ml) of the *Allium* species belonging to the subgenus *Rhizirideum* against all the plant pathogenic fungi

Seven members belonging to the subgenus *Rhizirideum* were investigated in the current study. Unfortunately, not much of an analytical information is available regarding these species. As seen in **Figure 5.10**, only *A. stellerianum* showed a rather low antifungal activity with a minimum inhibitory concentration less than 10mg/mL towards all the phytopathogenic fungi except for *Mucor hiemalis*.

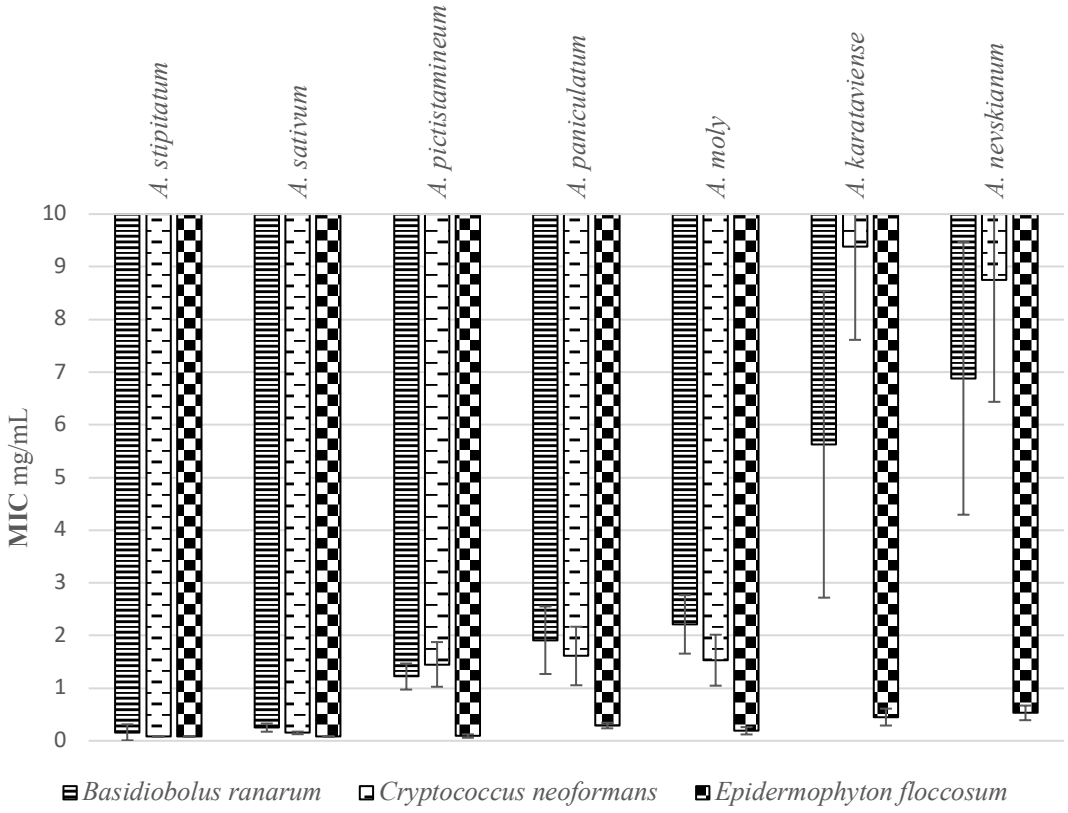


Figure 5.11 A diagram comparing MIC (mg/ml) gained from the antifungal activity tests of the seven *Allium* species against dermatophytes

Comparing the results gained from the antifungal activity tests of seven effective species against human pathogenic fungi demonstrated significant results as well. In this regard, it can be mentioned that *A. stipitatum* with a total average MIC of 0.11mg/mL proved to be the most effective *Allium* species towards the tested dermatophytes. *A. sativum* also proved to be effective by demonstrating a MIC of 0.16mg/mL. Although *A. sativum* seems to be less effective than *A. stipitatum* (subgenus *Melanocrommyum*) regarding micro-dilution as well as disk diffusion test, but in case of testing the effect of volatile compounds via double dish chamber, *A. sativum* showed an average ZOI of 67mm, as well as a complete growth inhibition of *Epidermophyton floccosum*. While *A. stipitatum* demonstrated an average ZOI of 45, having no effect on the growth of *Basidiobolus ranarum*. *A. pictistamineum* and

A. paniculatum subsp. *paniculatum* both from the subgenus *Allium* also had a very remarkable effect toward the human pathogens. They both showed an average MIC less than 2mg/mL (0.9mg/mL and 1.3mg/mL subsequently). More detailed results can be seen in **Figure 5.11**.

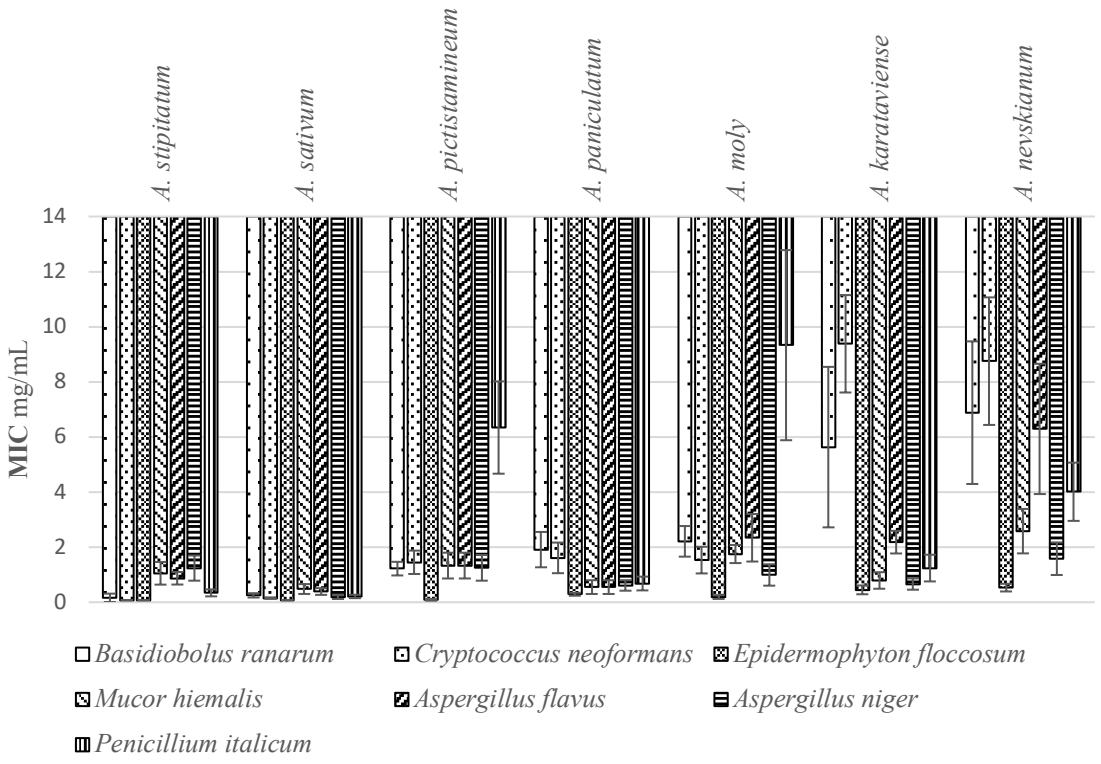


Figure 5.12 A diagram comparing MIC (mg/ml) between plant and human pathogen fungi

In **Figure 5.12** the comparison of seven effective species against dermatophytes as well as phytopathogenic fungi can be seen. Although *A. sativum* has always been assumed to be a powerful antimicrobial plant, this diagram proves that this *Allium* species may not be the only one with significant effects, and *A. stipitatum*, *A. pictistamineum* and *A. paniculatum* subsp. *paniculatum* are quite comparable with *A. sativum*.

By comparing the results obtained from the three different assays, a rather good correlation between them can be observed. Results obtained from PDA micro-dilution susceptibility testing confirmed that *A. sativum* from the subgenus *Allium*, with the total average MIC of 0.25mg/mL, represents the highest anti-fungal activity against all the tested human and plant pathogenic fungi. This is in good agreement with former results [54].

A. stipitatum belonging to the subgenus *Melanocrommyum* with a total average minimum inhibitory concentration of 0.55mg/mL also exhibited a significant effect towards all the

pathogens. As mentioned in chapter 4.7, the antifungal properties of this *Allium* are due to its bioactive pyridine-N-oxides.

A. paniculatum subsp. *paniculatum* and *A. pictistamineum*, both also belonging to subgenus *Allium*, showed a significant effect toward the selected pathogens with overall averages MIC of 0.89mg/mL and 1.86mg/mL, respectively. It can be assumed that these effects are related to relatively high amounts of alliin as well as further sulfur compounds with allyl rests. Although the entire average MIC of A. *ampeloprasum* and A. *vineale* were higher than those mentioned above, but they exhibited anti-fungal activity against all tested fungal samples with the inhibitory concentration less than 10mg/mL. Both, *A. ampeloprasum* as well as *A. vineale* show significant amounts of isoalliin besides alliin [153] giving less active mixed thiosulfinates after alliinase reaction as described above.

A rather similar outcome was obtained by the disk diffusion method. *A. stipitatum* gave the best results with a ZOI of 35mm, followed by *A. sativum* with a ZOI of 30mm. *A. paniculatum* subsp. *paniculatum* also demonstrated a ZOI of 16mm. It should also be mentioned that these three *Allium* species showed efficacy against all tested pathogenic fungi.

Thiosulfinates, as well as most of the less complex degradation products, are volatile. Selective antifungal activity of the VOCs has been tested by the double-dish chamber assay. Again, the best activity indicated by the largest ZOI was obtained for *A. sativum* additionally with 58mm. Fungal growth of all examined pathogens was likewise repressed by *A. paniculatum* subsp. *paniculatum* giving ZOI of 38mm. among all the tested fungi these two *Allium* species were the only samples effective not only against plant pathogens but also opposed to dermatophytes.

According to previous studies, garlic has been shown to inhibit the growth of a variety of microorganisms, not only bacteria but also fungi and viruses [54, 55]. As a result of this, determining new species which have a similar effect, like *A. stipitatum* (Mu-sir) and *A. paniculatum* subsp. *paniculatum*, proves the importance of further studies with regard to *Allium* family. Also, for these species, allicin and other allyl-sulfides, as well as pyridine N-oxides in case of *A. stipitatum*, can be expected as the main active principle. However, differences between the disc diffusion test and the double-dish chamber test revealed that there are more active compounds in the ethyl acetate extracts as only volatile sulfur

compounds. Possible candidates are high molecular sulfur compounds [157] and/or flavonoids and saponins [55]. Although more work is required to solve this problem.

Interestingly, nearly half of the tested bulb extracts showed only a little or no antifungal activity: Subgenus *Allium*: *A. atroviolaceum*, *A. rotundum* subsp. *rotundum, A. sphaerocephalon* subsp. *sphaerocephalon*; subgenus *Amerallium*: *A. cernuum*, *A. scorzonerifolium* var. *xericiense*; subgenus *Anguinum*: *A. victorialis*; subgenus *Butomissa*: *A. ramosum*; subgenus *Cepa*: *A. altaicum*, *A. cornutum*, *A. schoenoprasum*; subgenus *Polyprason*: *A. platyspathum* subsp. *amblyophyllum*, *A. talassicum*; subgenus *Melanocrommyum*: *A. atropurpureum*, *A. cyrilli*, *A. hollandicum*, *A. jesdianum* subsp. *angustitepalum*, *A. jesdianum* subsp. *jesdianum*, *A. macleanii*, *A. rosenorum*; subgenus *Retuculatobulbosa*; *A. strictum*; subgenus *Rhizirideum*: *A. denudatum*, *A. lusitanicum*, *A. nutans*, *A. rubens*, *A. senescens*, *A. spirale*. The phytochemical composition of many of these species has been recently investigated [153]. Out of these data it can be assumed that species with a relatively high content of methiin and a low content of alliin exhibit weak antifungal activity. With the exception of *A. obliquum*, this seems to be the case for the subgenera *Polyprason* and *Rhizirideum*. Wild species of the subgenus *Cepa* are characterized by a relatively high amount of methiin and isoalliin. Also, this combination seems to be not very favorable in terms of antifungal activity. In case of subgenus *Melanocrommyum,* it is believed that the formation of dithiopyrrole, the red dye, highly affects their antifungal activity, in a way that the species forming this compound showed absolutely no effect toward pathogenic fungi.

In conclusion, aggregated data showed acceptable agreement between the microorganism's susceptibility results based on the micro-dilution susceptibility testing method and disk diffusion test, while the overall levels of agreement between the MICs obtained by the micro-dilution method and the corresponding ZOIs measured via double-dish chamber were arguable in some cases. For instance, *A. carinatum, A. rotundum, A. campanulatum, A. jodanthum* and *A. stellerianum* all demonstrated MIC <10mg/mL at least toward 3 out of 4 tested fungi, albeit exhibiting no efficacy in regard to volatile compounds. However, better correlations were noted for *A. cepa aggregatum* group, *A. moly*, *A. rupestre, A. scorodoprasum, A. sibthorpianum* and *A. tuberosum*, all of them being not very active. These species mainly contain methiin and isoalliin [153].

Based on the here presented results, numerous EtOAc-extractions of *Allium* spp. have antifungal activity and might be promising candidates for 'biological' treatment of fungal-associated plant diseases. Most promising seems to be species from the subgenus *Allium* harboring about 560 species names, many of them invalid or unresolved. Species showing greatest antifungal effectiveness should be selected for further investigations regarding unknown cysteine sulfoxides. For they are believed to be the potential future drug candidates.

6. SUMMARY

In this thesis we investigated 51 members of the genus *Allium* belonging to 9 subgenera against 4 plant pathogenic fungi and 3 human pathogens (dermatophytes). The plant pathogenic fungi under consideration were *Mucor hiemalis, Aspergillus flavus*, *A. niger* and *Penicillium italicum*. The human pathogens of interest were *Epidermophyton floccosum*, *Cryptococcus neoformans,* and *Basidiobolus ranarum*.

To achieve the desired results, we employed different approaches, namely, broth microdilution method, agar microdilution method, disk diffusion method and double-dish chamber. Dilution series of ethyl acetate extracts obtained from *Allium* bulbs were tested on the above-mentioned fungi.

Among the tested *Allium* samples, *A. sativum* from the subgenus *Allium*, with the total average MIC of 0.3mg/mL, represents the highest anti-fungal activity against all the tested human and plant pathogenic fungi. *A. stipitatum* belonging to the subgenus *Melanocrommyum* with a total average minimum inhibitory concentration of 0.6mg/mL also exhibited a significant effect towards all the pathogens. *A. paniculatum* subsp. *paniculatum*, also belonging to subgenus *Allium*, showed a significant effect toward the selected pathogens with overall average MIC of 0.9mg/mL.

A rather similar outcome was obtained by the disk diffusion method. *A. stipitatum* gave the best results with a ZOI of 35mm, followed by *A. sativum* with a ZOI of 30mm. *A. paniculatum* subsp. *paniculatum* also demonstrated a ZOI of 16mm.

Regarding the double dish chamber method, the largest ZOI was obtained for *A. sativum* additionally with 58mm. Fungal growth of all examined pathogens was likewise repressed by *A. paniculatum* subsp. *paniculatum* giving ZOI of 38mm. among all the tested fungi these two *Allium* species were the only samples effective, not only against plant pathogens, but also opposed to dermatophytes.

E. floccosum showed the highest susceptibility, while *P. italicum* demonstrated greater resistance towards *Allium* extracts and miconazole.

Results indicate that extractions of *Allium* spp. have antifungal activity and might be promising in treatment of fungal-associated diseases. The effect seems to be partially related to volatile sulphur compounds. Nevertheless, other compounds like saponins and flavonoids must be considered.

7. ZUSAMMENFASSUNG

Die Gattung *Allium* als eine der größten Gattungen der Monokotyledone enthält mehr als 900 Arten aus 15 Untergattungen. Arten der Gattung *Allium* werden seit der Antike nicht nur als Gewürze oder Gemüse, sondern auch aufgrund ihrer medizinischen Eigenschaften eingesetzt und auch als Ziergewächse verwendet.

In dieser Studie wurden 51 Mitglieder der Gattung *Allium*, die zu 9 Untergattungen gehören, gegen 4 pflanzenpathogene Pilze, d. h. *Mucor hiemalis*, *Aspergillus flavus*, *A. niger* und *Penicillium italicum*, sowie 3 humanpathogene Erreger (Dermatophyten), d. h. *Epidermophyton floccosum*, *Cryptococcus neoformans* und *Basidiobolus ranarum*, getestet.

Zur Durchführung der Studie wurden folgende Methoden zur Bestimmung des antifungalen Aktivität verwendet: agar microdilution method, disk diffusion method und double-dish chamber. An den oben genannten Pilzen wurden Verdünnungsreihen von Ethylacetat-Extrakten aus *Allium*-knollen getestet.

Unter den untersuchten *Allium*-Proben hat *A. sativum* aus der Untergattung *Allium* mit einem durchschnittlichen MIC von 0,3mg/mL die höchste antimykotische Aktivität gegen alle getesteten human- und pflanzenpathogenen Pilze. *A. stipitatum*, der zur Untergattung *Melanocrommyum* gehört, zeigte mit einer durchschnittlichen minimalen

Hemmkonzentration von 0,6mg/mL ebenfalls eine signifikante Wirkung gegen alle Erreger. *A. paniculatum* subsp. *paniculatum*, aber ebenfalls zur Untergattung *Allium* gehört, zeigte eine signifikante Wirkung auf die ausgewählten Erreger mit einer durchschnittlichen MIC von 0,9mg/mL.

Ein ähnliches Ergebnis wurde mit der disk diffusion method erzielt. Die besten Ergebnisse lieferte *A. stipitatum* mit einer ZOI von 35mm, gefolgt von *A. sativum* mit einer ZOI von 30mm. *A. paniculatum* subsp. *paniculatum* zeigte auch ein ZOI von 16mm.

Bei der double-dish chambber wurde für *A. sativum* zusätzlich die größte ZOI mit 58mm erreicht. Das Pilzwachstum aller untersuchten Erreger wurde ebenfalls durch *A. paniculatum* subsp. *paniculatum* gibt ZOI von 38mm. Unter allen untersuchten Pilzen waren diese beiden *Allium*-Arten, die nicht nur gegen Pflanzenpathogene, sondern auch gegen Dermatophyten wirksam waren.

E. floccosum zeigte die höchste Sensibilität, während *P. italicum* eine größere Resistenz gegen *Allium*-Extrakte und Miconazol zeigte.

Die Ergebnisse zeigen, dass die Extrakte von *Allium* spp. eine antimykotische Wirkung haben, und können bei der Behandlung von pilzassoziierten Krankheiten eingesetzt werden. Der Effekt scheint teilweise mit flüchtigen Schwefelverbindungen zusammenzuhängen, aber auch andere Verbindungen wie Saponine und Flavonoide müssen berücksichtigt werden.

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Erklärung

Ich versichere, dass ich meine Dissertation:

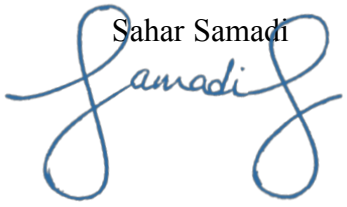
„Effectiveness of Bulb Extracts of the Genus *Allium*“

selbständig ohne unerlaubte Hilfe angefertigt und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen bedient habe. Alle vollständig oder sinngemäß übernommenen sind Zitate als solche gekennzeichnet.

Die Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

Marburg, den.....

Sahar Samadi



Curriculum Vitae

Name	Samadi
Vorname	Sahar
Staatsangehörigkeit	deutsch/iranisch
Geburtsdatum	05.07.1984
Geburtsort, -land	Teheran, Iran
09.1990 − 07.1995	Grundschule, Teheran, Iran
09.1995 − 07.1998	weiterführenden, Teheran, Iran
09.1998 − 07.2002	Gymnasium, Teheran, Iran
09.2002 − 06.2006	B.Sc., Biologie, Phytowissenschaften, Fachbereich Biologie, Universität Teheran, Teheran, Iran
09.2006 − 09.2009	M.Sc., Phytowissenschaften, Ökologie und Systematik, Fachbereich Biologie, Universität Teheran, Teheran, Iran
09.2009 − 09.2011	Wissenschaftliche Mitarbeiterin, Iranian Research Institute of Plant Protection, Teheran, Iran
11.2011 − Heute	Ph.D., Naturwissenschaften, Fachbereich Pharmazie, Philipps-Universität Marburg, Deutschland
01.2013 − 12.2015	Wissenschaftliche Mitarbeiterin, Fachbereich Pharmazie, Philipps-Universität Marburg, Deutschland
06.2017 − 10.2017	Weiterbildung zum Clinical Research Associate, Pharmaakademie, Leipzig, Deutschland
03.2018 − Heute	Quality assurance associate, PAION AG, Aachen, Deutschland